

**The Exploitation of Host Iron Sources by
Candida albicans during Oral Infection**

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1. Summary/ Zusammenfassung

1.1. Summary

Iron is an essential element for almost all organisms, from microbes to multicellular animals. Using high affinity iron-binding molecules, higher organisms can sequester virtually all free iron, causing a natural resistance to infection known as “nutritional immunity”. Therefore, pathogenic microbes are forced to utilise iron from host molecules during infection. For example, within the oral cavity, extracellular iron is mostly bound to lactoferrin found in saliva whilst intracellular iron is stored in association with ferritin. Although ferritin is abundant in epithelial cells, only one bacterial species (*Neisseria meningitidis*) has yet been shown to use ferritin indirectly as an iron source within infected epithelial cells by manipulating the cellular machinery.

Candida albicans is a polymorphic yeast which belongs to the normal microbial flora of human beings. The fungus exists as a harmless commensal on mucosal surfaces in healthy individuals but can cause several types of infections in predisposed patients, ranging from superficial to life-threatening disease. *C. albicans* possesses three known iron uptake systems: (i) uptake and usage of iron from haemoglobin, mediated by a haemoglobin receptor (Rbt5) and a haem oxygenase (Hmx1); (ii) siderophore iron utilisation mediated by the receptor Sit1 and (iii) free iron and transferrin iron uptake, mediated by the reductive iron uptake system.

During oral infections, *C. albicans* must be able to exploit the host iron resources. Since it was observed that the ferritin concentration within oral epithelial cells was directly related to their susceptibility to damage by *C. albicans*, it was hypothesized that host ferritin may be used as an iron source by this organism. In agreement with this, *C. albicans* was shown to grow on agar at physiological pH with ferritin as the sole iron source. In contrast, the baker's yeast *Saccharomyces cerevisiae* was unable to grow under these conditions. A screen of mutants lacking components of each of the three iron acquisition systems showed that only the reductive pathway is involved in ferritin iron utilization. The *Δftr1* mutant - which lacks a high affinity iron permease - grew with free iron, haemoglobin, but not with ferritin as the sole source of iron. The fact that growth with ferritin was enhanced when the initial pH of the medium was low, suggested that pH plays a crucial role in the release of iron from ferritin. Indeed, *C. albicans* was only able to use ferritin as an iron source under conditions which

permitted acid production and acidification of the surrounding environment. Using fluorescent stained anti-ferritin antibodies, it was shown that the vast majority of hyphal, but not yeast forms of *C. albicans* cells bound ferritin. Furthermore, electron microscopy analysis showed ferritin molecules localised on the hyphal cell wall. Because only hyphae displayed ferritin binding, mutants unable to form hyphae ($\Delta ras1$ and $\Delta cph1/efg1$) were tested for ferritin binding. $\Delta ras1$ and $\Delta cph1/efg1$ mutants were both completely unable to bind ferritin. To further investigate this observation, the ferritin binding potential of the $\Delta hgc1$ mutant was analysed. This mutant cannot form true hyphae but still expresses hyphal-specific genes. Yeast or pseudohyphae of the $\Delta hgc1$ mutant were able to bind ferritin under laboratory hyphae-inducing conditions, suggesting that candidate genes encoding putative ferritin receptors should be up-regulated in wild type and $\Delta hgc1$ cells, but should be unaltered or down-regulated in the $\Delta ras1$ mutant. Using *C. albicans* microarrays and RNA from wild type, $\Delta ras1$ and $\Delta hgc1$ cells, a total of 22 genes were identified with an expression profile indicative of a putative ferritin receptor. Three of these genes (*ECE1*, *HYR1* and *ALS3*) are known to encode cell surface localized, hyphal-specific proteins - as expected for a ferritin receptor. The corresponding knockout mutants of these three genes were then tested for their ability to bind ferritin. Both, the $\Delta ece1$ and the $\Delta hyr1$ mutants efficiently bound ferritin. In contrast, the $\Delta als3$ mutant completely lost its ability to bind ferritin. An $\Delta als3+ALS3$ revertant strain reconstituted the wild type ferritin binding phenotype. Additionally, deletion of *ALS3* caused growth defects on agar plates with ferritin as the sole iron source and a *S. cerevisiae* strain expressing Als3 was able to bind ferritin. Finally, binding of ferritin to hyphal surfaces not only occurred with exogenously added purified ferritin *in vitro*, but also during interactions of *C. albicans* with epithelial cells. Hyphae of the $\Delta als3$ mutant invading epithelial cells did not show ferritin accumulation, while invading wild type and revertant strains displayed dense layers of ferritin on hyphal surfaces. The essential roles of Als3 and Ftr1 for iron acquisition from ferritin during infection are supported by the fact that both $\Delta als3$ and $\Delta ftr1$ mutants lost their ability to damage epithelial cells.

In summary, this study suggests that *C. albicans* can use ferritin as an iron source via direct binding to Als3 on the surface of hyphae; iron is then released by acidification and uptake is facilitated by the reductive pathway. This is the first study which shows that a pathogenic microbe can directly use ferritin as an iron source.

1.2. Zusammenfassung

Für nahezu alle Lebewesen, von Mikroben bis zu vielzelligen Tieren, gehört Eisen zu den essentiellen Mineralstoffen. Höhere Organismen können über hochaffine Eisenbindepoteine nahezu das gesamte frei verfügbare Eisen sequestrieren und dadurch eine Form der Immunität gegen Infektionen aufbauen, die als „Ernährungsimmunität“ („nutritional immunity“) bezeichnet werden kann. Aus diesem Grund müssen pathogene Mikroorganismen während der Infektion Wirtsmoleküle als Eisenquelle nutzen können. So liegt in der Mundhöhle Eisen extrazellulär hauptsächlich an Lactoferrin im Speichel gebunden vor, während intrazelluläres Eisen mit Ferritin assoziiert ist. Obwohl Ferritin in Epithelzellen in großen Mengen vorkommt, ist bisher nur ein Mikroorganismus bekannt, der Ferritin zumindest indirekt nutzen kann: *Neisseria meningitidis* beeinflusst gezielt die wirtseigenen Abbauprozesse, um an das gebundene Eisen zu gelangen.

Candida albicans ist eine polymorphe Hefe, die zu der normalen mikrobiellen Flora des Menschen gehört. In gesunden Individuen tritt sie als Kommensale auf Schleimhäuten in Erscheinung, kann aber in entsprechend prädisponierten Patienten oberflächliche bis lebensbedrohende Infektionen hervorrufen. Drei Eisenaufnahmesysteme sind bei *C. albicans* bekannt: (1) Aus Hämoglobin über einen Hämoglobinrezeptor (Rbt5) und eine Hämoxygenase (Hmx1), (2) aus Siderophoren über den Rezeptor Sit1 und (3) über Aufnahme von freiem Eisen oder von Transferrin durch das reduktive System.

Während einer oralen Infektion muß *C. albicans* die Eisenvorräte des Wirtes nutzen können. Tatsächlich zeigte sich, dass die Ferritinkonzentration in Epithelzellen direkten Einfluß auf das Ausmaß der Schädigung durch *C. albicans* hat. Es konnte also vermutet werden, dass Ferritin eine Eisenquelle für diesen Pilz ist. In Übereinstimmung damit wächst *C. albicans* bei physiologischen pH-Werten auf Agar mit Ferritin als einziger Eisenquelle – im Gegensatz zu der Hefe *Saccharomyces cerevisiae*, die unter diesen Bedingungen nicht wachsen kann. Versuche mit Mutanten, denen jeweils einzelne Komponenten der drei Eisenaufnahmesysteme fehlen, haben gezeigt, dass nur der reduktive Weg für die Nutzung von Eisen aus Ferritin benötigt wird. Die *Afr1*-Mutante, bei der eine hochaffine Eisenpermease fehlt, wuchs zwar mit freiem Eisen und Hämoglobin, aber nicht mit Ferritin als Eisenquelle. Je niedriger der pH-Wert des verwendeten Mediums war, desto besser war auch das Wachstum. Diese Tatsache deutete darauf hin, dass der pH-Wert für die Freisetzung des Eisens aus Ferritin eine wichtige Rolle spielt. Tatsächlich konnte *C. albicans* Ferritin nur dann nutzen, wenn die Bedingungen die Ansäuerung des Mediums erlaubten. Mit fluoreszenzmarkierten Anti-

körpern konnte weiterhin gezeigt werden, dass Ferritin zwar von der überwältigenden Mehrzahl der Hyphen, nicht aber von Hefen gebunden wird. Elektronenmikroskopische Aufnahmen zeigten ebenfalls gebundenes Ferritin an den Zellwänden der Hyphen. Wegen dieser Spezifität wurden Mutanten untersucht, die keine Hyphen mehr bilden können. *Δras1* und *Δcph1/efg1* waren nicht mehr in der Lage, Ferritin zu binden. Deshalb wurde die Fähigkeit zur Ferritinbindung auch bei der Mutante *Δhgc1* untersucht, die keine echten Hyphen mehr bildet, aber trotzdem hyphenspezifische Gene exprimiert. Hefen und Pseudohyphen der *Δhgc1* Mutante konnten unter hypheninduzierenden Bedingungen Ferritin binden. Daraus konnte gefolgert werden, dass die Gene für mögliche Rezeptoren im Wildtyp und in der *Δhgc1* Mutante heraufreguliert sein sollten, während sie in der *Δras1* Mutante unverändert blieben oder herunterreguliert würden. Über eine Expressionsanalyse mit *C. albicans*-Microarrays und der RNA von Wildtyp, *Δras1* und *Δhgc1* Mutante konnten 22 Gene identifiziert werden, deren Expressionsprofil mit dem eines hypothetischen Ferritinrezeptors übereinstimmte. Drei dieser Gene (*ECE1*, *HYR1* und *ALS3*) kodieren für hyphenspezifische Oberflächenproteine, wie es für den Ferritinrezeptor erwartet wurde. Deshalb wurden Mutanten aller drei Gene auf ihre Ferritinbindung untersucht. Sowohl die *Δece1* als auch die *Δhyr1* Mutante konnte Ferritin binden. Im Gegensatz dazu hatte die *Δals3* Mutante diese Fähigkeit verloren. Die Revertante *Δals3+ALS3* zeigte wieder die Bindefähigkeit des Wildtyps. Weiterhin führte die Deletion von *ALS3* zur Wachstumshemmung auf Agar mit Ferritin als Eisenquelle, und ein *S. cerevisiae*-Stamm, der Als3 heterolog exprimiert, konnte Ferritin binden. Außerdem war die Bindefähigkeit nicht auf *in vitro* zugeführtes Ferritin beschränkt, sondern konnte auch bei der Interaktion mit Epithelzellen gezeigt werden. Während die *Δals3* Mutante bei der Invasion von Epithelzellen keine Anreicherung von Ferritin auf der Oberfläche zeigte, waren die Hyphen sowohl von Wildtyp als auch von Revertante dicht mit Ferritinmolekülen überzogen. Die essentielle Rolle von Als3 und Ftr1 für die Eisenaufnahme aus Ferritin während einer Infektion zeigte sich auch daran, dass beide Mutanten ihre Fähigkeit zur Schädigung von Epithelzellen verloren haben. Zusammenfassend wird mit dieser Arbeit ein Modell vorgeschlagen, nach dem *C. albicans* Ferritin als Eisenquelle nutzen kann, indem es direkt an Als3 auf der Hyphenoberfläche gebunden wird. Das Eisen wird dann durch Ansäuerung freigesetzt und über den reduktiven Weg aufgenommen. Damit wurde zum ersten Mal eine direkte Nutzung von Ferritin als Eisenquelle durch einen Mikroorganismus gezeigt.

2. Introduction

2.1. Iron and iron homeostasis in the host

2.1.1. The role of iron in biological systems

Iron, the fourth most plentiful element in the Earth's crust, has the following characteristics: it can vary its oxidation state, reduction potential and electronic spin configuration depending on its ligand molecule (Crichton and Pierre, 2001). These properties make iron an essential cofactor for a variety of different types of proteins such as cytochromes and other haem-containing proteins. Iron-containing enzymes are required for numerous biochemical processes, including oxygen transport, cellular respiration and metabolism, drug metabolism and DNA synthesis (Welch *et al.*, 2001). In aqueous environments, iron switches from the relatively soluble ferrous state (0.1 M at pH 7.0) to the very insoluble ferric form (10^{-18} M at pH 7.0) (Andrews *et al.*, 2003). However, at acidic pH the predominant form is Fe^{2+} (ferrous iron) and at neutral/alkaline pH, ferrous iron autoxidation occurs to form Fe^{3+} (ferric iron) (Kosman, 2003).

2.1.2. Iron proteins: haemoglobin, transferrin, lactoferrin and ferritin

Although non-toxic in its insoluble ferric state, soluble ferrous iron can be toxic due to its participation in the generation of hydroxyl radicals via the Fenton reaction ($\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^\cdot$) (Halliwell and Gutteridge, 1984). Hydroxyl radicals can depolymerise polysaccharides, cause DNA strand breaks, inactivate enzymes and initiate lipid peroxidation (McCord, 1996). To resolve this problem, evolutionary pressures have selected highly conserved iron regulation systems. In vertebrates, iron is almost totally bound to iron proteins, preventing it from catalysing free radical cascades and blocking its availability to pathogens. In humans, iron is present at around 40-50 mg/kg (Crichton and Charleaux-Wauters, 1987) with 66% of the total body iron circulating in the blood, coupled to haemoglobin (Evans *et al.*, 1999).

Haemoglobin, the oxygen-binding protein of red blood cells, transports oxygen from the lungs to tissues and carbon dioxide back to the lungs. It is a compact globular protein of

approximately 64 kDa, comprised of two pairs of polypeptide chains, termed α and β ; the resulting structure is that of an $\alpha_2\beta_2$ tetramer (reviewed in Marengo-Rowe, 2006). In each polypeptide chain nestles one haem prosthetic group. Haem can only bind oxygen if ferrous iron (Fe^{2+}) is present. Furthermore, oxidation of the ferrous to the ferric form yields methaemoglobin, which does not bind oxygen. Thus, each haemoglobin molecule possesses four haem groups containing one ferrous ion per prosthetic group (Evans *et al.*, 1999).

The transferrin family constitutes another class of iron protein. This family consists of a group of monomeric glycoproteins of approximately 90 kDa with high-affinity for ferric iron ($K_d \sim 10^{-22}$ M) (Aisen *et al.*, 1978). They function by sequestering extracellular iron, hence avoiding the damaging effects of iron-catalysed free radical cascades and exerting bacteriostatic effects through iron chelation. There are three major members of the transferrin family. Ovotransferrin, the first characterized member, is the major component of egg white (Cohn *et al.*, 1949).

The second member of the transferrin family is itself named transferrin. Found in serum, transferrin can bind ferric iron and transport it from sites of iron absorption and storage to sites of utilization (Fletcher and Huehns, 1968). The majority of transferrin is synthesized in the liver and only about 3 mg iron (approximately 0.1% of the total body iron) is bound to transferrin (Evans *et al.*, 1999). In healthy human beings, transferrin is only 30% saturated with iron (Han, 2005), thus the free iron concentration in serum is about 10^{-18} M (Bullen *et al.*, 1978). Transferrin possesses two iron-binding sites and bicarbonate is required for its ability to bind iron (Fletcher and Huehns, 1968). Because of its low-affinity for ferrous iron, acidification accelerates iron removal from transferrin (Morgan, 1979). The proposed mechanism by which transferrin releases iron is via binding to the transferrin receptor coupled with acidification to pH 5.6 (Bali and Aisen, 1992).

Finally, similar to transferrin, lactoferrin possesses two ferric iron-binding sites and requires bicarbonate for iron binding (Jolles *et al.*, 1976). It is present in body fluids such as milk, saliva, tears and serum, and is released from neutrophils upon degranulation (Vorland, 1999). Independent of its iron binding ability, lactoferrin contains a defensin-like peptide and has microbicidal activity against microorganisms such as *Candida albicans*, *Streptococcus mutans*, *Vibrio cholerae* and enterobacteria (Yamauchi *et al.*, 1993; Vorland *et al.*, 1999; Tanida *et al.*, 2001; Ueta *et al.*, 2001; Ward *et al.*, 2002).

The iron storage protein, ferritin, is present in a number of diverse organisms, from bacteria to higher eukaryotes. It is found in virtually all human cells; however, the liver, spleen and bone marrow contain the highest levels of the protein. In healthy human adults about 30% of total body iron is present in ferritin (Fleming and Wood, 1995). Ferritin plays a key role in maintaining intracellular iron homeostasis by capturing and “saving” free iron from toxic reactions (Torti and Torti, 2002). Consequently, deletion of the ferritin H subunit gene is lethal in mice (Ferreira *et al.*, 2000). Although the majority of the ferritin in the human body is intracellular, low levels (20 – 300 µg/l) can be found circulating in blood (Harrison and Adams, 2002). Serum ferritin is an important index of body iron, but its function remains unknown (Lipschitz *et al.*, 1974; Torti and Torti, 1994). Furthermore, hyperferritinaemia (high levels of ferritin in serum) is known to be associated with a number of medical conditions with non-HIV systemic infections and malignancies reported as the major aetiological factors (Le Page *et al.*, 2005).

Structurally, ferritin is composed of 24 subunits forming a protein nanocage (spherical shape) with around a 12 nm outer diameter and an inner cavity of about 8 nm in diameter and is capable of accommodating around 4500 iron atoms (Harrison *et al.*, 1991). The spatial arrangement of the subunits results in the formation of two different types of channel: the fourfold channels (six per protein) are hydrophobic and are unlikely to provide a route for the movement of metal ions; the threefold channels (eight per protein) are hydrophilic and it is proposed that these channels provide the route for ferrous iron to enter the inner cavity (Ford *et al.*, 1984).

Human ferritin is composed of two types of subunits, H (21 kDa) and L (20 kDa) (Arosio *et al.*, 1978). Depending on the tissue type, the ratio of H to L subunits can vary from predominantly L in liver and spleen, to predominantly H in heart and kidney (Arosio *et al.*, 1976). The H subunit possesses ferroxidase activity and thus influences the uptake of iron by ferritin (Sun and Chasteen, 1992). The L subunit plays a role in iron-core nucleation and confers stability to assembled ferritin molecules (Levi *et al.*, 1992).

When apoferritin (empty ferritin) is assembled, ferrous iron oxidation and ferricoxyhydroxide formation are proposed to occur on the inner surface of the protein at di-iron ferroxidase centres located within the H-ferritin subunits ($2 \text{ Fe}^{2+} + \text{O}_2 + 4 \text{ H}_2\text{O} \rightarrow 2 \text{ FeOOH}_{(\text{core})} + \text{H}_2\text{O}_2 + 4 \text{ H}^+$) (Bauminger *et al.*, 1991; Yang and Chasteen, 1999). Following significant core growth (~ 50 – 300 iron atoms), the site of ferrous iron

oxidation changes to the surface of the growing ferricoxyhydroxide crystal and the stoichiometry increases from 2 to 4 ferrous iron/oxygen ($4 \text{ Fe}^{2+} + \text{O}_2 + 6 \text{ H}_2\text{O} \rightarrow 4 \text{ FeOOH}_{(\text{core})} + 8 \text{ H}^+$), corresponding to ferrous iron autooxidation (Xu and Chasteen, 1991; Yang and Chasteen, 1999).

Two mechanisms of iron release from ferritin have been proposed. Kidane and co-workers, using three different cell lines (hepatic, Caco2 and K562 cells), showed that inhibiting lysosomal proteases – with leupeptin and chymostatin – and avoiding lysosomal acidification – with chloroquine – decreased the rate of ferritin iron release and ferritin degradation. This demonstrated that ferritin iron release in cell culture requires lysosomal activity (Kidane *et al.*, 2006). Additionally, in the same study, the authors showed that cellular ferritin levels were dramatically decreased by co-incubation with the iron chelator desferoxamine and were increased by co-incubation with ferric ammonium citrate.

The second proposed mechanism by which iron is removed from ferritin involves the action of natural reductants or chelators, in combination with localized and reversible unfolding of the ferritin pores by physiological concentrations of urea. The unfolding of the ferritin pores would facilitate the entry of reductants/chelators into the protein shell with subsequent ferrous iron release (Theil *et al.*, 2006; Liu *et al.*, 2007). In agreement with this model, reductants/chelators such as thioglycolic acid, ascorbate, aceto- and benzohydroxamic acids are capable of releasing iron from the ferritin core (Joo *et al.*, 1990; Laulhere *et al.*, 1996; Galvez *et al.*, 2005). Underscoring the importance of pH in the release of iron from ferritin, this process was increased at pH 5.2 in comparison to pH 7.4 (Galvez *et al.*, 2005). Moreover, the quaternary structure of ferritin is dissociated *in vitro* under pH 2.0 and the structure is recovered at pH 7.4 (Domínguez-Vera, 2004).

2.1.3. Cellular iron uptake and storage

Normal daily iron requirements are provided by either dietary iron or via the recycling of senescent blood cells. The translocation of this iron and its availability are tightly regulated. Extracellular ferrous iron is oxidized by ceruloplasmin and the generated ferric iron is bound by transferrin. The ligation of transferrin to two ferric iron molecules (holotransferrin, hTF) increases by two-fold its affinity for the transferrin receptor (TFR), present on the surface of virtually all mammalian cells. Following endocytosis of the hTF-TFR complex into the early endosome, acidification to pH 5.6

results in the release of iron from holotransferrin. The released ferrous iron is then transported to the cytoplasm by the divalent metal transporter (DMT1) and either used for cellular metabolism or stored within ferritin. The resulting apotransferrin (transferrin without iron) is recycled to the cell surface and released at physiological pH (7.4) (Bali and Aisen, 1992; Klausner and Rouault, 1996; Rouault, 2003).

The concentrations of ferritin and the transferrin receptor are regulated at the post-transcriptional level based on mRNA sequences, known as “iron-regulatory elements” (IREs). Cytoplasmic iron-regulatory proteins (IRPs) mediate this post-transcriptional regulation via binding to the IRE sites of ferritin and transferrin receptor mRNA. When the cytosolic iron concentration is low, IRPs bind to IRE sequences at the 5'-untranslated region of ferritin mRNA, inhibiting the association of the 40S ribosomal subunit and blocking mRNA translation. Concurrently, the binding of IRPs to IRE sequences in the 3'-untranslated region of transferrin receptor mRNA protects the transcript from degradation by endonucleases. This increase in transferrin receptor synthesis leads to increased transferrin/iron uptake. When cellular iron levels are high, binding of IRPs to IREs is reduced. Consequently, ferritin mRNA is translated, whilst the transferrin receptor mRNA is degraded by endonucleases (Rouault and Klausner, 1996; Torti and Torti, 2002).

Non-transferrin iron uptake mechanisms have also been reported. Free ferric iron can be reduced at the plasma membrane by the ferric reductase, DCYTB (duodenal cytochrome-b-like reductase) and transported inside the cells by the permease DMT1 (Schaible and Kaufmann, 2004). In macrophages, haem-bound iron (e.g. haemoglobin) is taken up by the cell through the haemoglobin scavenger receptor, CD163 (Hentze *et al.*, 2004). Moreover, neutrophils and epithelial cells secrete neutrophil-gelatinase-associated lipocalin (NGAL) that complexes iron, probably through the binding of lipophilic chelators (Goetz *et al.*, 2002; Kaplan, 2002). Yang and co-workers demonstrated that iron loaded NGAL is internalised in endosomes and, like transferrin, is recycled (Yang *et al.*, 2002). Finally, a mouse ferritin receptor has recently been identified and characterized. The ferritin receptor (Tim2) mediates specific binding to and endocytosis of H-ferritin. This finding, for the first time, demonstrates the existence of a ferritin receptor-mediated, transferrin-independent pathway for cellular ferritin accumulation (Chen *et al.*, 2005).

2.2. Iron and microbial infection

2.2.1. The importance of iron for infection: iron limitation and iron overload

Like their host, the majority of microorganisms require iron for growth. *Borrelia burgdorferi*, the causative agent of Lyme disease, is the only known pathogen that does not require iron for cellular metabolism. The genome sequence of *B. burgdorferi* contains no known iron-protein-encoding genes and severe iron limitation does not affect its growth (Posey and Gherardini, 2000).

Apart from *B. burgdorferi*, all known pathogenic microorganisms need to compete with the host for iron. Consequently, iron acquisition and regulation, and connections with metabolism and virulence, have been reported for several species of pathogenic bacteria and fungi. In pathogenic *Escherichia coli* strains, more than 90 genes involved in iron acquisition along with several other cellular functions, such as chemotaxis, respiration, DNA synthesis, glycolysis and the tricarboxylic acid cycle are co-regulated by the global iron-dependent regulator *FUR* (**F**erric-**U**ptake **R**egulator protein) (Andrews *et al.*, 2003). Furthermore, Fur homologues have been found to regulate iron-responsive gene expression in a large number of Gram-negative bacteria and in some Gram-positive bacteria (Andrews *et al.*, 2003).

The transcriptional repressor *DtxR* in *Corynebacterium diphtheriae* regulates iron acquisition and expression of the diphtheria toxin in response to iron. Similar to Fur, the apoprotein form (protein without iron) of DtxR loses its DNA-binding affinity under iron limitation. Consequently, iron uptake and toxin genes are up-regulated (Schmitt *et al.*, 1997; Andrews *et al.*, 2003).

In the fungus *Cryptococcus neoformans* the *Cryptococcus* iron regulator (Cir1) regulates not only the expression of genes involved in iron uptake, but also genes involved in calcium metabolism, the ability to grow at 37°C, glucan synthesis, sterol biosynthesis and two key virulence factors: capsule formation and melanin production (Jung *et al.*, 2006).

Siderophores are low molecular weight chelators (less than 1 kDa) with high-affinity for ferric iron. In bacteria and fungi, the production and secretion of siderophores are positively controlled by iron limitation (Andrews *et al.*, 2003; Haas, 2003). The gene *sidA* – necessary for hydroxamate-type siderophore synthesis – is essential for virulence in *Aspergillus fumigatus* (Schrettl *et al.*, 2004; Hissen *et al.*, 2005).

Such coordinated regulation indicates that sensing the low iron content of the host environment is a key signal for pathogenic microbes to initiate adaptation to the host.

Although iron limitation serves as a signal for the expression of virulence factors and iron uptake systems, iron overload in the host – owing to nutritional, hereditary or therapeutic reasons – can also exacerbate several infectious diseases, including yersiniosis, salmonellosis, and tuberculosis (Schaible and Kaufmann, 2004). Additionally, in a mouse model of cryptococcosis, iron overload exacerbates meningoencephalitis (Barluzzi *et al.*, 2002).

2.2.2. Iron sources and strategies used by pathogens

To access and use host iron, microbial pathogens have developed three main strategies: (i) the secretion of siderophores that sequester host iron and/or the up-take of siderophores, (ii) direct or indirect iron utilisation from host iron proteins and (iii) the overproduction of acidic end-products that release iron from host proteins.

The purified siderophore desferri-exochelin, secreted by *Mycobacterium tuberculosis*, rapidly removes iron from transferrin and lactoferrin, but at a slower rate from ferritin (Gobin and Horwitz, 1996). The filamentous fungus, *Aspergillus fumigatus*, produces two siderophores (triacetylfusarinine C and ferricrocin) both capable of removing iron from transferrin and these iron acquisition mechanisms have been shown to be important for the survival of *A. fumigatus* in human serum (Hissen *et al.*, 2004). Finally, *C. neoformans* cannot produce siderophores, but can use siderophores from other species (Jung and Kronstad, 2008).

Desferal is a siderophore (desferoxamine) produced by actinomycetes, used in the treatment of iron-loaded patients. Although desferal prevents iron overload in humans, treatment with this iron chelator predisposes the patients to *Mucor* infections, because desferoxamines sequesters iron from transferrin, supplying previously unavailable iron to the fungus (Boelaert *et al.*, 1993; Boelaert *et al.*, 1994).

Specific, receptor-mediated binding of transferrin and lactoferrin has been demonstrated in *Neisseria* species. These receptors are located in the bacterial outer membrane and are induced under iron starvation. After binding to the receptors, iron is stripped from transferrin or lactoferrin by an as-yet undetermined mechanism, and the apoproteins (iron-free) are released extracellularly rather than being internalised and degraded (reviewed in Perkins-Balding *et al.*, 2004). Iron acquisition from transferrin can also be

host-specific. For example, *Actinobacillus pleuropneumoniae* is harmless to humans but virulent in pigs. This host-specificity of *A. pleuropneumoniae* is due to its binding specificity to porcine transferrin (Jacobsen *et al.*, 2005).

Access to host haem from haemoglobin requires haemolysis and subsequent binding of haemoglobin. Some bacteria can also secrete haemophores that bind extracellular haemoglobin and mediate its delivery to surface receptors. Once haem has reached the cytoplasm, it is degraded by haem oxygenase, releasing the complexed iron (reviewed in Genco and Dixon, 2001).

Ferritin is an extremely robust and stable protein which seems to be resistant to all known microbial activities. In fact, *Neisseria meningitidis* is the only microorganism that has so far been shown experimentally to exploit holoferritin as an indirect iron source during interaction with host cells. The bacteria can trigger degradation of cytosolic ferritin within infected epithelial cells by manipulating the host cellular machinery and lysosomal activity, and utilises the resultant cytosolic iron (Larson *et al.*, 2004).

In addition to these examples of specific pathogen-host factor binding events, some bacteria can also influence their environment in a much more general way in order to acquire iron. For example, under iron limitation, *Staphylococcus aureus* increases production of lactate, resulting in acidification of the surrounding environment and, consequently, the release of iron from transferrin (Friedman *et al.*, 2006).

2.3. *Candida albicans*

2.3.1. The genus *Candida*

Taxonomically, *Candida* is a member of the family Candidaceae, in the order Saccharomycetales, in the class Hemiascomycetes, in the phylum Ascomycota, in the kingdom Fungi. Approximately 150 species belong to the genus *Candida*, but only 13 species are able to cause infection in humans. *C. albicans*, *C. dubliniensis*, *C. famata*, *C. glabrata*, *C. guilliermondii*, *C. inconspicua*, *C. kefyr*, *C. krusei*, *C. lusitaniae*, *C. parapsilosis*, *C. tropicalis*, *C. utilis* and *C. viswanathii* form the group of known pathogenic species (Odds, 1988; Calderone, 2002).

Few *Candida* species (such as *C. albicans*, *C. dubliniensis*, *C. glabrata* and *C. parapsilosis*) are part of the normal microbial flora of the skin and mucosal surfaces in more than 50% of the total human population (Soll, 2002). The most frequently isolated species is *C. albicans*, comprising more than 70% of the total isolates (Ruhnke, 2002). Lay and Russel reported that 6% of newborn children were orally colonized by *Candida* spp.; by one month of age, colonization increased to 79% (Lay and Russel, 1977).

2.3.2. The polymorphic fungus *C. albicans*

C. albicans can grow in a number of morphological forms. *C. albicans* yeast cells are ovoid-shaped cells, 4-6 x 6-8 μM (Calderone, 2002). However, given certain environmental cues, *C. albicans* yeast cells are able to undergo morphological transitions and form one of two types of distinct filaments: hyphae or pseudohyphae. Hyphae are highly elongated cells with parallel walls, which lack constrictions at the septa. In contrast, pseudohyphae consist of chains of elliptical cells with constrictions at the septa (Sudbery *et al.*, 2004; Kumamoto and Vines, 2005a). Further morphological forms can arise during colony switching (opaque phase cells) or growth under certain environmental conditions (chlamydospores) (Sudbery *et al.*, 2004).

2.3.3. Growth and genetics of *C. albicans*

In vitro, *C. albicans* is able to grow/survive under extreme conditions of pH (ranging from 2.5 to 10) and temperature (ranging from 5°C to 46°C) (Odds, 1988). Furthermore, yeast growth occurs at 30°C under acidic pH (< pH 6) and with high cell concentrations (> $10^7/\text{ml}$). Hyphal growth is triggered at 37°C under neutral/alkaline pH (> pH 7) and with low cell concentrations (< $10^7/\text{ml}$) (Odds, 1988).

C. albicans is a diploid fungus which lacks a classical sexual cycle. Sequencing of the *C. albicans* genome was completed in 1998 at the Stanford Genome Technology Center using the clinical isolate SC5314. The annotation of *C. albicans* genes is based primarily on sequence comparison with the baker's yeast *Saccharomyces cerevisiae*. The haploid karyotype of *C. albicans* consists of 8 chromosomes (1-7 and R) (Magee *et al.*, 1992). The genome consists of 14.88 Mb, comprising 6,354 genes and of these,

19.2% (1,218 genes) encode proteins unique to *C. albicans* (Braun *et al.*, 2005). One genetic peculiarity of *C. albicans* (and other members of the so-called CUG-clade) is its codon usage. In the translation of tRNA_{CAG}, serine is decoded instead of leucine (Santos *et al.*, 1993). Technically, this uncommon codon usage provides a challenge for genetic manipulation and heterologous protein expression.

2.3.4. The pathogenic fungus *C. albicans*

In most humans, *C. albicans* exists as a harmless commensal; however, it is able to cause disease when the equilibrium of the microbial flora is disturbed (e.g. during long term antibiotic treatment) or if the immune system of the host is compromised. There are two main forms of infection: superficial infections of the skin and mucosa, and invasive candidiasis, where the fungus can disseminate throughout the blood system and infect virtually every organ (Calderone, 2002).

Candida species are the most frequent cause of invasive fungal infections in humans. Recent studies have shown that *Candida* spp. are now the third most frequently isolated organisms from nosocomial bloodstream infections and *C. albicans* is the most frequently isolated species (approximately 50%) (Perlroth *et al.*, 2007). Furthermore, up to 90% of untreated HIV-infected individuals suffer from oral *Candida* infections (Ruhnke, 2002) and most women experience at least one vaginal yeast infection in their lifetime, with *C. albicans* being the most frequent isolate (Sobel, 2002).

Unlike certain bacterial pathogens that express a single toxin (e.g. *Clostridium tetani*) as a major virulence factor, the fungal pathogen *C. albicans* possesses a range of determinants that contribute to virulence. And rather than constitutive expression of this repertoire of virulence factors in the host, it seems as if particular virulence determinants are of pathogenic significance only in certain host niches. In the sections below, a selection of factors contributing to virulence, with an emphasis on iron uptake will be discussed.

2.4. *C. albicans* virulence factors

2.4.1. Hyphal formation

Although all three morphological forms (yeast, pseudohyphae and hyphae) can be observed in organs colonized by *C. albicans*, the ability to form hyphae *in vivo* has been considered an important virulence attribute of *C. albicans*. Most mutants known to be unable to form hyphae under laboratory conditions are attenuated in virulence (Lo *et al.*, 1997). However, hyphal formation itself is associated with the expression of a subset of genes, which encode virulence factors not involved in hyphal formation *per se*, confounding the true role of hyphal morphogenesis in *C. albicans* virulence. Such hyphal-associated genes are normally expressed during hyphal morphogenesis, but the lack of these genes does not influence hyphal development. Comprising a set of hyphal-specific genes, with known function, are: *HWP1*, *ALS3*, *SAP4*, *SAP5* and *SAP6* (their function will be described in the next sections). Additionally, *ECE1*, *HYR1*, *RBT1*, *RBT4* and *DDR48* are also included in the set of hyphal-specific genes, but the function of these genes remains to be clarified (Kumamoto and Vines, 2005b).

The discovery of the hyphal-specific G1 cyclin-related protein (Hgc1) (Zheng *et al.*, 2004) was a step forward in determining the role played by hyphal morphogenesis in *C. albicans* virulence. Deletion of *HGC1* results in cells that grow normally in the yeast form but fail to produce hyphae. Despite this, the $\Delta hgc1$ mutant cells still expressed three hyphal-associated genes (*HWP1*, *ECE1* and *HYR1*) (Zheng *et al.*, 2004). The fact that the $\Delta hgc1$ mutant displays defective hyphal morphogenesis, without affecting the expression of hyphal-associated genes, and is attenuated in a mouse model of systemic infection, provided support for the long-held belief that hyphal formation *per se* is important for virulence.

A number of pathways govern hyphal development, depending on the environmental stimuli. For example, the putative transcription factor *CZF1* plays a key role in regulating morphogenesis under embedded conditions (growth within an agar matrix) but is not required for hyphal development under other hyphal induction conditions (Brown *et al.*, 1999). *CPH1*, the *C. albicans* homologue of the *STE12* transcription factor from *S. cerevisiae* is involved in filamentous growth in certain media and is partially involved in filamentous growth within agar (Liu *et al.*, 1994; Giusani *et al.*, 2002). *EFG1* encodes a basic helix-loop-helix transcription factor that is required

for hyphal development under most laboratory conditions (Stoldt *et al.*, 1997). However *Δefg1* mutant cells are hyperfilamentous under embedded conditions (Giusani *et al.*, 2002). The *Δcph1/Δefg1* double null mutant is more defective in hyphal development than either single mutant, indicating that the two genes have partially overlapping functions. Moreover, this double null mutant fails to cause mortality following intravenous inoculation of mice (Lo *et al.*, 1997). Upstream of the *CPH1* and *EFG1* pathways is the GTP-binding protein Ras1. The *Δras1* mutant cells are defective in hyphal formation in serum-containing media and under embedded conditions (Feng *et al.*, 1999; Maidan *et al.*, 2005). Additionally, this mutant is attenuated in virulence in a mouse model of systemic infection (Leberer *et al.*, 2001).

Tec1 is a member of the TEA/ATTS family of transcription factors and one of the downstream effectors of Efg1. The *Δtec1* mutant cells exhibit suppressed filamentation in serum-containing media and show reduced virulence in a systemic model of candidosis (Schweizer *et al.*, 2000).

Downstream from Tec1 is a zinc finger protein (Bcr1) required for biofilm formation but not hyphal development. Bcr1 activates cell surface protein and adhesin genes such as *ALS1*, *ALS3*, *HWP1*, *RBT5*, *HYR1* and *ECE1*. Although Bcr1 is essential for biofilm formation *in vitro* and *in vivo*, it is not required for systemic infection in a mouse model (Nobile and Mitchell, 2005; Nobile *et al.*, 2006).

In contrast to the regulators described above, *TUP1* encodes a transcriptional repressor of hyphal development in *C. albicans* (Braun and Johnson, 1997). To exercise its function, Tup1 is complexed to at least two different DNA-binding proteins (Rfg1 and Nrg1) (Braun *et al.*, 2001; Kadosh and Johnson, 2001; Khalaf and Zitomer, 2001; Murad *et al.*, 2001). The Tup1/Rfg1 and Tup1/Nrg1 complexes repress partially overlapping sets of hyphal-associated genes (Kadosh and Johnson, 2005). Furthermore, Knight and co-workers demonstrated that Tup1 regulates the expression of *CFL95* (encoding a ferric reductase) and *FTR1* (encoding an iron permease), relating filamentation with high-affinity iron uptake (Knight *et al.*, 2002).

2.4.2. Adhesion

Adhesion to host cells is a prerequisite for *C. albicans* to colonise the host. This virulence determinant is mediated primarily by adhesins: molecules that bind host factors.

The *ALS* (Agglutinin-Like-Sequence) gene family, comprised of eight members, encodes glycosylphosphatidylinositol (GPI)-anchored cell wall glycoproteins (reviewed in Hoyer, 2001). Als3 promotes adherence to both human umbilical vein endothelial cells (HUVEC) and buccal epithelial cells (Zhao *et al.*, 2004). Recently, a new function of Als3 was reported. Phan and co-workers have shown that Als3 is a fungal invasin that mimics host cell cadherins and induces endocytosis by binding to N-cadherin on endothelial cells and E-cadherin on oral epithelial cells (FaDu) (Phan *et al.*, 2007). Additionally, $\Delta als3$ mutant cells are unable to damage endothelial cells and oral epithelial cells and are strongly attenuated in their ability to damage epithelial tissue in an *in vitro* model of oral infections (Reconstitute Human Epithelium – RHE) (Zhao *et al.*, 2004; Phan *et al.*, 2007). Surprisingly, the $\Delta als3$ mutant is fully virulent in a mouse model of systemic infection (Scott Filler, personal communications).

Als1 mediates adherence of *C. albicans* to endothelial cells, but not to buccal epithelial cells (Fu *et al.*, 2002; Zhao *et al.*, 2004). Additionally, Als1 is not required for damage of endothelial and epithelial cells but is involved in damaging epithelial tissue (RHE model) (Zhao *et al.*, 2004; Phan *et al.*, 2007). In contrast to $\Delta als3$, the $\Delta als1$ mutant displays reduced virulence in a mouse model of systemic infection (Fu *et al.*, 2002).

Deletion of *ALS5*, *ALS6* or *ALS7* results in increased adherence to endothelial cells and buccal epithelial cells in comparison to wild type. In the RHE model, however, mutant and control strains caused a similar degree of tissue damage (Zhao *et al.*, 2007a).

Another well-studied adhesin of *C. albicans* is the hyphal-specific cell wall protein, Hwp1. This GPI-anchored cell wall protein plays a role in adherence to buccal epithelial cells (Staab *et al.*, 1999), but is not required for adherence to endothelial cells (Tsuchimori *et al.*, 2000). Hwp1 is a substrate for keratinocyte transglutaminase, mediating a covalent binding of *C. albicans* to host cells (Staab *et al.*, 1999). Additionally, $\Delta hwp1$ mutant cells are moderately compromised for virulence following intravenous inoculation in mice (Sundstrom *et al.*, 2002).

2.4.3. Secreted hydrolases

Secreted aspartic proteases (Saps) serve as important virulence factors by degrading proteins such as components of the extracellular matrix and proteins involved in host defence, enhancing *C. albicans* pathogenicity (Naglik *et al.*, 2003). At least 10 different genes encode Saps and the members of this family are expressed under different laboratory conditions (Naglik *et al.*, 2003). Sap1-6 and Sap8 are secreted, whilst Sap9/10 are cell surface localised (Naglik *et al.*, 2003; Albrecht *et al.*, 2006). The localisation of Sap7 is unknown. Albrecht and co-workers have demonstrated that Sap9/10 contain GPI consensus sequences and anchor to either the membrane or cell wall. Here, Sap9/10 are required for maintaining cell surface integrity, play a role in adhesion to buccal epithelial cells and are required for epithelial tissue damage (RHE model) (Albrecht *et al.*, 2006).

Additionally, the proteases encoded by the hyphal-specific genes *SAP4*, *SAP5* and *SAP6* are important for invasion of parenchymal tissue following intraperitoneal inoculation of the mouse (Felk *et al.*, 2002). In contrast, the proteases Sap1-3, but not Sap4-6, are associated with mucosal infections (De Bernardis *et al.*, 1999).

Phospholipases represent a second class of hydrolytic enzymes produced by *C. albicans*. It has been demonstrated that *C. albicans* possesses phospholipase A-, B-, C- and D-activity (Niewerth und Korting, 2001). Only the members of the phospholipase B-family (*PLB1-5*) have been shown to be secreted (Mavor *et al.*, 2005). Furthermore, *Δplb1* mutant cells have reduced virulence in both systemic and intragastric models of infection (Ghannoum, 2000; Mukherjee *et al.*, 2001).

The third family of extracellular hydrolases is comprised of 10 highly homologous genes encoding lipases (*LIP1-10*) (Fu *et al.*, 1997; Hube *et al.*, 2000). Hube and co-workers have shown that *LIP5/6/8/9* are expressed during intraperitoneal infection, suggesting a role for lipases in *C. albicans* virulence (Hube *et al.*, 2000). Recently, the first lipase knockout mutant (*LIP8*) was produced. The *Δlip8* mutant is reduced in virulence in a mouse model of systemic infection, supporting the role of these extracellular hydrolases in *C. albicans* virulence (Gacser *et al.*, 2007).

2.4.4. pH sensing

As a successful pathogen, *C. albicans* must adapt to diverse environmental conditions, including dramatic variations in environmental pH. The response to a neutral-to-alkaline pH change is controlled in part by the Rim101 signal transduction pathway. Neutral-to-alkaline environments are sensed at the plasma membrane, by two putative membrane sensors, Dfg16 and Rim21 (Davis, 2003; Thewes *et al.*, 2007). Following signal transduction, involving other components of this pathway (the full molecular mechanisms of which have not yet been clearly established), the zinc finger transcription factor Rim101 is activated and acts to induce alkaline-responsive gene expression (Davis, 2003).

Bensen and co-workers used a microarray approach to analyse the transcriptional response of *C. albicans* wild type and $\Delta rim101$ mutant to acidic and alkaline pH. The authors showed that Rim101 positively regulates a subset of genes at alkaline pH, including those involved in iron acquisition (Bensen *et al.*, 2004). At alkaline pH, iron is present in the insoluble form (Fe^{3+}), resulting in an iron-limited environment. Consistent with these findings, it was demonstrated that both Rim101 and Dfg16 are required for growth in iron-limited media at alkaline pH, but not at acidic pH (Bensen *et al.*, 2004; Thewes *et al.*, 2007).

Additionally, both $\Delta rim101$ and $\Delta dfg16$ mutants had strongly reduced virulence in a mouse model of systemic infection (Davis *et al.*, 2000; Thewes *et al.*, 2007). In contrast, cells lacking *DFG16* showed no decrease in epithelial tissue damage in comparison to wild type cells (Thewes *et al.*, 2007).

2.4.5. Iron uptake

Because iron is an essential element for both the host and *C. albicans*, iron uptake during infection is considered a virulence factor. Pre-treatment of endothelial cells with the iron chelator phenanthroline reduced damage by *C. albicans*, and loading endothelial cells with iron reversed this protective effect (Fratti *et al.*, 1998). Moreover, endocytosis of *C. albicans* by phenanthroline-treated cells was reduced in comparison to non-treated cells, while adherence of the fungus to chelator-treated endothelial cells was slightly enhanced. Finally, the cyto-protective effects of iron chelation were not due

to inhibition of the synthesis of reactive oxygen intermediates, demonstrating that the protective effect was indeed mediated by iron sequestration.

In a mouse model of systemic candidosis, intravenous injection with colloidal iron (60 mg/kg weight), for three consecutive days before intravenous inoculation of *C. albicans* yeast cells (10^7 cells) significantly increased the mortality rate of mice: within 28 days of infection, 40% of mice without iron administration died, while 80% mortality was observed amongst iron loaded animals (Abe *et al.*, 1985).

Lan and co-workers examined the transcriptional profiles of *C. albicans* under high-iron or iron-limited conditions. Genes that were up-regulated under iron limitation included those associated with iron acquisition, as well as genes which affect mitochondrial function, cell-surface properties and virulence-related secreted hydrolases. Among these genes, the authors identified *SFUI*, which encodes a homologue of an *Ustilago maydis* (a maize pathogenic fungus) transcriptional repressor of siderophore uptake/biosynthesis. Comparisons between *C. albicans* wild type and $\Delta sfu1$ mutant strains revealed 139 potential target genes of Sfu1, many of which are iron-responsive (Lan *et al.*, 2004).

To acquire iron, *C. albicans* possesses distinct iron uptake systems for different iron-containing substrates. There are independent systems for acquiring iron from siderophores, from haem and from varied ferric iron chelates.

2.5. The three known iron uptake systems of *Candida albicans*

2.5.1. Iron reductive pathway

To use free iron from the environment, from transferrin and perhaps from other so far unknown iron sources, *C. albicans* uses the reductive uptake system. This system is located in the plasma membrane and consists of three activities. Two surface ferric reductases, which are able to reduce insoluble extracellular ferric (Fe^{3+}) ions into soluble ferrous (Fe^{2+}) ions, have been described (Hammacott *et al.*, 2000; Knight *et al.*, 2002; Knight *et al.*, 2005). In addition, a further thirteen homologous genes, putatively encoding ferric reductases have been identified in the *C. albicans* genome (Baek and Davis, 2008). Since reduced ferrous iron generated by surface reductase activity can be toxic, due to the spontaneous generation of free radicals, Fe^{2+} ions are subsequently

oxidized to Fe^{3+} and transported into the cell by a protein complex consisting of a multicopper oxidase and an iron permease. In total, the *C. albicans* genome contains five genes with homology to multicopper oxidase genes (Eck *et al.*, 1999). Due to the copper requirement of the oxidase activity, the intracellular copper transporter Ccc2 is essential for function of the reductive pathway (Weissman *et al.*, 2002). Two proteins with iron permease activity are encoded by two highly homologous genes, with different affinities for iron ions and with differential regulation. The high-affinity iron permease gene *FTR1* is induced upon iron deprivation, and the low affinity *FTR2* is induced when higher levels of iron are available. Moreover, from all components of the reductive pathway, only Ftr1 has been shown to be crucial for *C. albicans* virulence in an experimental animal model of infection (Ramanan and Wang, 2000).

2.5.2. Siderophore uptake

Heterologous siderophores are utilized via the siderophore transporter, Sit1 (Heymann *et al.*, 2002; Hu *et al.*, 2002). Although *C. albicans* siderophore production had been demonstrated by biochemical assays in earlier studies (Holzberg and Artis, 1983; Ismail *et al.*, 1985), genes encoding factors of a possible siderophore biosynthetic pathway have not been discovered in the *C. albicans* genome (Haas, 2003; Lan *et al.*, 2004). However, Sit1 can mediate the uptake of a range of siderophores from other organisms and from other iron complexes (Ardon *et al.*, 2001; Heymann *et al.*, 2002; Lesuisse *et al.*, 2002; Bernier *et al.*, 2005). A mutant lacking Sit1 is attenuated in virulence in the Reconstituted Human Epithelium (RHE) model of infection, but has wild type virulence in a mouse model of systemic infection (Heymann *et al.*, 2002).

2.5.3. Iron acquisition from haemoglobin

The first step in haemoglobin (Hb) iron utilisation by *C. albicans* and other pathogens *in vivo* has been proposed to be through the lysis of erythrocytes. For this, the fungus possesses a haemolytic factor, probably attached to its surface. However, the mechanism by which *C. albicans* causes haemolysis and the genetic basis of this mechanism remain unknown (Luo *et al.*, 2001). Following its release, the uptake of Hb is mediated via specific Hb-receptors. Weissman and Kornitzer first identified the Hb-receptor gene family, two of which (*RBT5* and *RBT51/PGA10*) have been characterised

in vitro as possessing Hb-receptor properties. The Hb-receptor gene family comprises the genes *RBT5*, *RBT51*, *WAP1/CSA1*, *CSA2* and *PGA7* (Weissman and Kornitzer, 2004). An $\Delta rbt5$ mutant displays wild type virulence in a mouse model of systemic infection and during rabbit corneal infection. This is probably due to compensatory mechanisms of other iron acquisition systems or due to the fact that other members of the gene family are overexpressed in the absence of *RBT5* (Weissman and Kornitzer, 2004).

To release iron from Hb, *C. albicans* possesses a cytoplasmic haem oxygenase. The mechanism by which the fungus transports haem from its surface to the cytoplasm remains unclear. *HXMI* – a gene with no other homologues in the *C. albicans* genome – encodes an active haem oxygenase, which degrades Hb to α -biliverdin. The role of *HXMI* in *C. albicans* virulence has not yet been determined. Interestingly, its expression is positively regulated by iron deprivation, by haem and by a temperature shift from 30 to 37 °C (Santos *et al.*, 2003). Furthermore, the $\Delta hmx1$ mutant was unable to grow under iron restriction. A role for haem oxygenase activity in the reductive pathway has not yet been established (Pendrak *et al.*, 2004).

In addition to its role as an iron source, a secondary function for Hb during candidosis has been proposed. The binding of Hb to the surface of *C. albicans* (discussed above), triggers the expression of putative surface receptors that enable the fungus to bind to fibronectin, laminin and fibrinogen (Yan *et al.*, 1998). Based on these findings, it has been suggested that the binding to Hb can stimulate *C. albicans* to bind to proteins of the host cell surface and the cellular matrix, favouring its invasion into host tissues (Pendrak *et al.*, 2004).

2.5.4. Host iron proteins used as iron sources by *C. albicans*

Several *in vitro* studies have analysed the interaction of *C. albicans* with host iron-binding proteins. For example, it has been shown that haemoglobin (described in 2.5.3) can be used as an iron source by *C. albicans in vitro*. Apotransferrin is known to inhibit *C. albicans* growth through iron chelation (Han, 2005; Lee and Han, 2006). In contrast, Knight and co-workers demonstrated that *C. albicans* can use transferrin as an iron source *in vitro* (Knight *et al.*, 2005). In this study it was shown that binding to transferrin and the reductive pathway are necessary for iron utilisation from transferrin

(Knight *et al.*, 2005). The authors speculated that monoferric transferrin (transferrin associated with only one iron molecule) could not be used as an iron source by *C. albicans*. Lactoferrin, as described above (section 2.1.2), contains a defensin-like peptide and has microbicidal activity against *C. albicans*. Thus far, no report has been published which describes the usage of lactoferrin as an iron source by this fungus. Finally, the intracellular iron storage protein ferritin seems to be very resistant to microbial attacks and its role as an iron source for *C. albicans* has not been reported. Furthermore, it remains unclear which iron sources are used during the different types of *C. albicans* infections and within different body sites.

2.6. Aims of this study

C. albicans possesses distinct uptake systems for iron-acquisition from different iron-containing substrates. There are independent systems for acquiring iron from siderophores, from haem and from varied ferric iron chelates. However, the natural sources of iron in different host niches and during different stages of infection remain unknown. This study aimed to investigate the natural iron sources and the iron acquisition systems used by *C. albicans* during oral infection. Because the ferritin content of oral epithelial cells (TR146 cell line) influenced damage of these cells by *C. albicans*, it was proposed that ferritin serves as an iron source for the fungus. Consequently, the main aim of this study was to investigate whether *C. albicans* can use ferritin as an iron source *in vitro* and during interaction with oral epithelial cells. Furthermore, assuming that ferritin can be used as an iron source, it was intended to elucidate the mechanism by which *C. albicans* can exploit this host iron storage protein.

3. Materials and Methods

3.1. Microorganisms

Table 3.1: *C. albicans* strains used in this study

Strain	Genotype	Reference
SC5314	wild-type	(Gillum <i>et al.</i> , 1984)
CAF2-1	<i>ura3::imm434</i> / <i>URA3</i>	(Fonzi and Irwin, 1993)
CAI-4+Clp10	<i>ura3::imm434/ura3::imm434</i> + Clp10 (<i>URA3</i>)	(Murad <i>et al.</i> , 2000)
$\Delta als3$	<i>ura3::imm434::URA3-IRO1/ura3::imm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG als3::ARG4/als3::HIS1</i>	(Nobile <i>et al.</i> , 2006)
$\Delta als3+ALS3$	<i>ura3::imm434::URA3-IRO1/ura3::imm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG als3::ARG4 ::ALS3/als3::HIS1</i>	(Nobile <i>et al.</i> , 2006)
$\Delta bcr1$	<i>ura3::imm434/ura3::imm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG pHis1 bcr1::ARG4/bcr1::URA3</i>	(Nobile and Mitchell, 2005)
$\Delta ccc2$	<i>ura3::imm434/ura3::imm434 ccc2::hisG/ccc2::hisG-URA3-hisG</i>	(Weissman <i>et al.</i> , 2002)
$\Delta cph1/efg1$	<i>ura3::imm434/ura3::imm434 cph1::hisG/cph1::hisG efg1::hisG/efg1::hisG-URA3-hisG</i>	(Lo <i>et al.</i> , 1997)
$\Delta dfg16$	<i>ura3::imm434/ura3::imm434 dfg16::hisG/dfg16::hisG + Clp10 (URA3)</i>	(Thewes <i>et al.</i> , 2007)
$\Delta ece1$	<i>ura3::imm434::URA3-RO1/ura3::imm434 ece1::hisG/ece1::hisG</i>	(Birse <i>et al.</i> , 1993)
$\Delta ftr1$	<i>ura3::imm434/ura3::imm434 ftr1::hisG/ftr1::hisG-URA3-hisG</i>	(Ramanan and Wang 2000)
$\Delta hgc1$	<i>ura3::imm434/ura3::imm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG hgc1::ARG4/hgc1::HIS1</i>	(Zheng <i>et al.</i> , 2004)
$\Delta hgc1+Clp10$	<i>ura3::imm434/ura3::imm434 arg4::hisG/arg4::hisG his1::hisG/his1::hisG hgc1::ARG4/hgc1::HIS1 + Clp10 (URA3)</i>	This study
$\Delta hyr1$	<i>ura3::imm434/ura3::imm434 hyr1::hisG/hyr1::hisG-URA3-hisG</i>	(Bailey <i>et al.</i> , 1996)
$\Delta ras1$	<i>ura3::imm434/ura3::imm434 ras1::hisG/ras1::hph</i>	(Feng <i>et al.</i> , 1999)
$\Delta ras1+Clp10$	<i>ura3::imm434/ura3::imm434 ras1::hisG/ras1::hph + Clp10 (URA3)</i>	This study
$\Delta rbt1$	<i>ura3::imm434/ura3::imm434 rbt1::hisG/rbt1::hisG-URA3-hisG</i>	(Braun <i>et al.</i> , 2000)
$\Delta rbt5$	<i>ura3::imm434/ura3::imm434 rbt5::hisG/rbt5::hisG-URA3-hisG</i>	(Weissman and Kornitzer, 2004)
$\Delta rim101$	<i>ura3::imm434/ura3::imm434 arg4::hisG/arg4::hisG HIS1::his1::hisG/his1::hisG rim101::ARG4/rim101::URA3</i>	(Wilson <i>et al.</i> , 1999)
$\Delta sap1-3$	<i>ura3::imm434/ura3::imm434 sap1::hisG/sap1::hisG sap2::hisG/sap2::hisG sap3::hisG/sap3::hisG-URA3-hisG</i>	(Felk <i>et al.</i> , 2002)
$\Delta sap4-6$	<i>ura3::imm434/ura3::imm434 sap6::hisG/sap6::hisG sap4::hisG/sap4::hisG sap5::hisG/sap5::hisG-URA3-hisG</i>	(Sanglard <i>et al.</i> , 1997)
$\Delta sit1$	<i>ura3::imm434/ura3::imm434 sit1::hisG/sit1::hisG-URA3-hisG</i>	(Heymann <i>et al.</i> , 2002)
$\Delta tec1$	<i>ura3::imm434 /ura3::imm434 tec1::hisG/tec1::hisG + pVEC (URA3)</i>	(Schweizer <i>et al.</i> , 2000)

Table 3.2. *S. cerevisiae* strains used in this study

Strain	Genotype	Reference
ATCC9763	wild-type	American Type Culture Collection
pADH1	<i>leu2 his3 trp1 ura3</i> + pADH1	(Sheppard <i>et al.</i> , 2004)
pALS1	<i>leu2 his3 trp1 ura3</i> + pALS1	(Sheppard <i>et al.</i> , 2004)
pALS3	<i>leu2 his3 trp1 ura3</i> + pALS3	(Sheppard <i>et al.</i> , 2004)
pALS5	<i>leu2 his3 trp1 ura3</i> + pALS5	(Sheppard <i>et al.</i> , 2004)

3.2. Preparation of low iron medium (LIM)

LIM medium is based on the Wickerham nitrogen base recipe (Wickerham, 1946) and modified by Eide and Guarente (Eide and Guarente, 1992). The stock solutions used for LIM medium preparation are described in Table 3.3.

Table 3.3. Composition of LIM

Stock	Fold conc.	Component	Stock conc. (M)	Final conc. (M)	Vol.
1	500	Na ₂ EDTA.2H ₂ O	5.0x10 ⁻¹	1.0x10 ⁻³	1ml
2	100	MgSO ₄ .7H ₂ O	5.0x10 ⁻¹	5.0x10 ⁻³	5ml
		NaCl	1.0x10 ⁻¹	1.0x10 ⁻³	
3	100	CaCl ₂ .2H ₂ O	1.0x10 ⁻¹	1.0x10 ⁻³	5ml
4	100	Uridine	4.0x10 ⁻²	4.0x10 ⁻⁴	5ml
		L-Histidine	5.0x10 ⁻²	5.0x10 ⁻⁴	
		L-Leucine	7.6x10 ⁻²	7.6x10 ⁻⁴	
		L-Lysine	7.0x10 ⁻²	7.0x10 ⁻⁴	
5	100	(NH ₄) ₂ SO ₄	3.8	3.8x10 ⁻²	5ml
6	100	KH ₂ PO ₄	1.0x10 ⁻¹	1.0x10 ⁻³	5ml
7	50	Na ₃ Citrate.2H ₂ O	1.0	2.0x10 ⁻²	10ml
8	20	D-glucose	2.2x10 ⁻¹	1.1x10 ⁻²	25ml
9	1000	d-Biotin	1.6x10 ⁻⁵	1.6x10 ⁻⁸	0,5ml
		Ca Pantothenate	1.7x10 ⁻³	1.7x10 ⁻⁶	
		myo-Inositol	1.0x10 ⁻²	1.0x10 ⁻⁵	
		Pyridoxin	2.0x10 ⁻³	2.0x10 ⁻⁶	
		Thiamin.HCl	1.0x10 ⁻³	1.0x10 ⁻⁶	
10	10000	H ₃ BO ₃	1.0x10 ⁻¹	1.0x10 ⁻⁵	50µl
		Cu ₂ SO ₄ .5H ₂ O	2.0x10 ⁻³	2.0x10 ⁻⁷	
		KI	5.0x10 ⁻³	5.0x10 ⁻⁷	
		MnCl ₂ .4H ₂ O	2.5x10 ⁻¹	2.5x10 ⁻⁵	
		Na ₂ MoO ₄ .2H ₂ O	1.0x10 ⁻²	1.0x10 ⁻⁶	
		ZnSO ₄ .7H ₂ O	3.3x10 ⁻¹	3.3x10 ⁻⁵	

The stock solutions were prepared from analytical-grade chemicals and ultra-pure water. The pH values of stocks 1 and 7 were adjusted to 8.0 and 4.2, respectively. Stock 10 was prepared in 0.1 M-HCl. Solutions were filter-sterilized with 0.2 µm cellulose nitrate filters (Schleicher & Schuell) and stored in polycarbonate bottles. To prepare the medium, the stock solutions were added in numerical order to 438.5 ml of ultra-pure water, filter-sterilized and stored in polycarbonate bottles. Polycarbonate bottles and

graduated cylinders were used to avoid iron contamination during preparation of stock solutions and medium. Finally, because concentrations of free iron can be increased by the photoreduction of ferric to ferrous iron in the presence of EDTA, media were prepared and inoculated in indirect light, all solutions containing EDTA were stored in the dark and cultures were incubated in the dark.

3.3. Fungal preculture conditions

C. albicans were grown in liquid YPD medium (1% yeast extract [Merck], 2% bactopectone [Difco], and 2% D-glucose [Roth]) in a shaking incubator at 30°C for 8 h. Subsequently, the cultures were diluted 1:1000 in LIM0 medium and incubated in a shaking incubator at 30°C overnight for iron starvation. For non-starved cells, precultures were incubated in YPD medium overnight at 30°C with shaking. Yeast cells were harvested by centrifugation, washed three times in filter sterilized ultra-pure water and counted using a haemocytometer.

3.4. Growth kinetics under iron limitation

C. albicans (SC5314) cells from an overnight YPD-preculture (see section 3.3) were inoculated (2×10^5 cells/ml) in 100 ml LIM medium (see Table 3.3) without addition of iron (LIM0), with 1 μ M ferric iron (LIM1) or 20 μ M ferric iron (LIM20). The cultures were incubated in a shaking incubator at 37°C and 150 rpm. Every hour, 1 ml of the culture was collected and the optical density (630 nm) was measured in duplicate for up to 15 hours incubation. Cell morphology was analysed microscopically using a Neubauer chamber. At least 100 cells per sample were counted and the percentages of yeast and hyphae cells were calculated.

To investigate possible intracellular iron storage in *C. albicans*, cells were inoculated (2×10^5 cells/ml) in Sabouraud medium containing increasing concentrations (as indicated in Figure 4.4) of bipyridine (extra- and intracellular iron chelator, Sigma-Aldrich) or bathophenanthrolineisulfonic acid disodium salt (BPS, extracellular iron chelator, Sigma-Aldrich). Cells incubated in Sabouraud medium without addition of chelator was used as control. Cultures were incubated in a shaking incubator at 37°C and 150 rpm. Every hour, 1 ml of the culture was collected and the optical density (630 nm) was measured in duplicate for up to 11 hours.

3.5. Hyphal growth under iron limitation

To analyse the effect of iron limitation on *C. albicans* hyphal formation and elongation, cells were grown in hyphae-inducing medium (RPMI 1640 medium [PAA]) with addition of the iron chelator apotransferrin (human transferrin without iron [Sigma-Aldrich]). Wild type (SC5313) cells from an overnight YPD-preculture were inoculated (2×10^5 cells/ml) in 20 ml RPMI 1640 medium containing 25 $\mu\text{g/ml}$ apotransferrin and 100 μM iron chloride (FeCl_3 ; Merck) in combination or separately, as indicated in Figure 4.2. Cells incubated in RPMI 1640 medium alone were used as a control. Cultures were incubated in a shaking incubator at 37°C and 150 rpm. After 6 h and 12 h of incubation, 1 ml of the culture was collected in a 1.5 ml microcentrifuge tube and 0.1% SDS (Roth) was added. Next the cell suspensions were sonicated using 5 cycles of 10 s each with a 30 s break between each cycle. It should be noted that these procedures were used to disrupt cell clumps. Following sonication, the cells were harvested by centrifugation. The cells (10 $\mu\text{l/sample}$) were analysed microscopically for percentage hyphae and hyphal length. At least 100 cells per sample were counted for determination of percentage hyphae. To calculate hyphal-length, at least 10 hyphae per sample were measured using AxioVision software (Zeiss).

3.6. Oral epithelial cells

The epithelial cell line TR146, derived from a squamous cell carcinoma of buccal mucosa (Rupniak *et al.*, 1985), was kindly provided by Cancer Research Technology, UK. TR146 cells were routinely grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; PAA). For experiments, epithelial cells were used between passages 10 to 20. Monolayers with 70-90% confluent cells in 24 well plates (TPP) were additionally incubated for 24 h under three different conditions: (1) RPMI 1640 with 50 μM bathophenanthrolinedisulfonic acid disodium salt (BPS; iron chelator [Sigma-Aldrich]); (2) RPMI 1640 with 10% FBS; (3) RPMI 1640 with 10% FBS and indicated concentrations of iron chloride. After 24 h incubation, monolayers were washed twice with phosphate-buffered saline without calcium or magnesium (PBS) and serum-free RPMI 1640 medium was added. Each well was infected with $\sim 10^6$ *C. albicans* cells and incubated for 8 h. Supernatants were removed for LDH measurements. All incubations were performed in a humidified incubator at 37°C in 5% CO_2 .

To monitor the ferritin content of cells, the uninfected monolayers were fixed with Roti[®]-Histofix 4% (Roth) and the ferritin content of the cells was visualized microscopically using immunofluorescence. Briefly, fixed monolayers were permeabilized through incubation with 0.1% Triton X-100 (Serva) for 15 min at room temperature and washed three times with PBS. Next, the samples were blocked using Image-iT[™] FX signal enhancer (Invitrogen) for 30 min at room temperature in a humidity chamber. Cells were again washed three times with PBS and incubated with rabbit anti-ferritin antibody (Sigma-Aldrich) coupled with dye DY-649 (Dyomics) diluted 1:1000 in PBS with 1% bovine serum albumin (BSA, Sigma-Aldrich) for 1 h at room temperature. Finally, cover-slips were washed three times with PBS, inverted and mounted on a microscope slide with ProLong[®] Gold Antifade Reagent (Invitrogen). The samples were analyzed in duplicates using a Leica DM 5500B microscope (Leica) and 10 randomly chosen fields per cover slip were photographed using a DFC 350 FX camera (Leica). A representative picture of each condition was selected.

3.7. Epithelial cell monolayer damage assay

Epithelial cell damage caused by *C. albicans*, was determined by measuring the release of lactate dehydrogenase (LDH) into the medium using a Cytotoxicity Detection Kit – LDH (Roche). The assays were performed according to the manufacturer instructions and the measurements were performed in duplicate. To measure epithelial cell damage, the following calculation was used: $100 \times (ECa-C1-C2)/(100L-C1) = \text{relative cytotoxicity (\%)}$. Absorbance measured at OD 490-600 directly correlates with LDH activity.

ECa = epithelial cells infected with *C. albicans*;

C1 = control 1 - uninfected epithelial cells;

C2 = control 2 - only *C. albicans*;

100L = 100% lysis (0.2% Triton-X 100, Serva).

Controls 1, 2 and 100% lysis were determined for each experiment.

3.8. High-affinity iron uptake assay

To investigate defects in high-affinity iron uptake, *C. albicans* cells were first starved of iron (see section 3.3) and serial dilutions were spotted on YPD agar (2% BactoTM agar, Difco) plates with or without 120 μ M BPS. Plates were incubated for 2 days at 37°C. Under these conditions, *C. albicans* wild type (SC5314) can grow (positive control strain), and an *Afr1* mutant lacking the high-affinity permease cannot (negative control strain).

3.9. Iron quantification

To quantify iron in solutions, the chaotropic agent guanidinium thiocyanate (Roth) was used. It binds iron, generating a red colour in the solution which can be monitored using a spectrophotometer. To generate a standard curve for iron quantification, a solution consisting of 1 M guanidinium thiocyanate diluted in 1 M HCl was mixed with increasing concentrations of ferric iron (2.5 μ M, 5 μ M, 10 μ M, 25 μ M, 50 μ M and 100 μ M) and absorbance was measured in triplicate at 447 nm. The R^2 value demonstrated that the method is reliable (Figure 4.7A).

3.10. Removal of free iron contamination from ferritin solution

To remove free iron contamination, the ferritin solution (holoferritin from horse spleen [Sigma-Aldrich]) was diluted 1:10 in dilution buffer (5 mM HEPES [Sigma-Aldrich]; 0.1 M NaCl [Roth]; pH 7.4) and passed through a Microcon YM-100 Centrifugal Filter Unit (cut off size 100 kDa [Millipore]). The retentate was collected in a new 1.5 ml microcentrifuge-tube. The dilution buffer was used to adjust the retentate to the original volume and the solution was again passed through a new column. This procedure was repeated 4 times. The eluate was used for iron quantification (described in section 3.9). For use in further experiments, the ferritin solution was diluted 1:10 in dilution buffer and passed once through the column, immediately prior to use.

3.11. Ferritin agar plates

To investigate whether *C. albicans* was able to grow with ferritin as the sole source of iron, 350 μ M BPS was added to the SD agar medium (6.7 g/l yeast nitrogen base, YNB [Difco]; 20 g/l D-glucose [Roth]; 20 g/l purified agar [Oxoid]). Additionally, HEPES buffer was added to the medium as indicated and the pH was adjusted to 7.4 or 5.0 as indicated. To prevent active acidification of the medium by the fungus, 20 g/l casamino acids (Difco) was used in place of D-glucose. Purified ferritin solution (see section 3.10) was plated out on agar surfaces at indicated concentrations. The respective incubation times and conditions are indicated in the figure legends.

3.12. Agar plates with pH indicator

To investigate whether *C. albicans* and *S. cerevisiae* were able to acidify the surrounding medium, 78 mg/l of pH indicator (bromocresol green [Sigma-Aldrich]) was added to the SD agar medium (6.7 g/l yeast nitrogen base, YNB; 20 g/l D-glucose; 20 g/l purified agar) and the pH was adjusted to 7.4 with NaOH (Roth). Approximately 50 iron-starved cells were spotted (in a 5 μ l volume) on agar and incubated for four days at 37°C under 5% CO₂ (*C. albicans*, SC5314) or 30°C (*S. cerevisiae*, ATCC9763).

3.13. Ferritin binding assay

C. albicans cells growing under iron limitation, as described above, were washed and enumerated. Approximately 5×10^5 cells were added per well in a 24 well plate (TPP) containing Poly-L-Lysine-coated (Biochrom AG) 12-mm diameter glass cover slips and 1 ml RPMI 1640. To induce hyphae, cells were incubated for 3 h at 37°C under 5% CO₂. Afterwards, the cells were washed once with PBS and incubated for 1 h in 1 ml PBS with 1% bovine serum albumin (BSA) and 100 μ g/ml ferritin. Subsequently, the cells were washed three times with PBS to remove non-bound ferritin and fixed with 500 μ l Roti[®]-Histofix 4%.

To test if viability is necessary for ferritin binding, *C. albicans* hyphae (3 h in RPMI 1640 at 37°C and 5% CO₂) were killed using two different approaches: either 1.5 h incubation at room temperature with 0.05% Thimerosal (Sigma-Aldrich) or two exposures to 0.5 Joules UV light in a UV-crosslinker (Vilber-Loumart). Complete

killing, without residual viability of cells was checked by plating the cells on YPD agar plates. After killing, the cells were incubated with ferritin and fixed as described above. The fixed cells were washed three times with PBS and incubated with rabbit anti-ferritin antibody coupled with dye DY-649 diluted 1:2000 in PBS with 1% BSA for 1 h at room temperature. Next, the cover slips were inverted and mounted on a microscope slide with ProLong® Gold Antifade Reagent (Invitrogen) and cells were visualized using a Leica DM 5500B microscope (Leica). Photomicrographs were taken using a DFC 350 FX camera (Leica). To quantify how many *C. albicans* cells bound ferritin, at least 100 cells per cover slip were counted and percent binding was calculated by counting the total number of cells and the number of cells displaying fluorescent signal. All binding assays were performed in duplicates. Cells incubated without ferritin were used as a negative control.

Because *S. cerevisiae* cells were detached during the washing steps described above, a different approach was used. Briefly, 5×10^5 cells were added per well in a 24 well plate containing Poly-L-Lysine-coated 12-mm diameter glass cover-slips in 1 ml RPMI 1640. The cells were incubated for 1 h at 30°C. Afterwards, the medium was removed and 250 µl PBS with 1% BSA and 25 µg/ml ferritin coupled with dye DY-649. After 15 min at 30°C, the cells were washed once with PBS, fixed, mounted and visualized under the microscope as described above for *C. albicans* cells.

3.14. Transmission electron microscopy

C. albicans wild-type cells (SC5314) were grown on poly-L-lysine-coated cover-slips (0.5 mm in diameter) in the presence or absence of 100 µg/ml ferritin for 6 h in RPMI 1640. Afterwards, the cells were washed with PBS four times to remove non-bound ferritin and then immersed in fixative – 4% formaldehyde (prepared from para-formaldehyde [Roth] and 0.1% glutaraldehyde [Roth] in 0.05 M HEPES) at room temperature. After three min the fixative was replaced with fresh fixative and stored at 4°C overnight. The samples were dehydrated in ethanol (Roth) by progressively lowering the temperature to -35°C and infiltrated with Lowicryl K4M resin (Polysciences) at -35°C (Fuchs *et al.*, 2003). The resin polymerization was carried out under UV light at -35°C for 24 h and for 10 h at 0°C. Ultra-thin sections (60-80 nm thick) were produced with an Ultracut S (Leica) and a diamond knife. Sections were collected on formvar filmed copper slot grids. Bright-field transmission electron

microscopy was performed with an EM902 (ZEISS) at 80 kV. Images were recorded with a 1k CCD camera (Proscan).

The iron distribution was calculated by recording three images, two directly below the ionisation edge of the iron atom to calculate the background, which was then subtracted from the image obtained at the ionisation edge of iron (L3, max = 718 eV).

The electron microscopy analysis was done in collaboration with Dr. Michael Laue at the Robert Koch Institute in Berlin.

3.15. Biological function of ferritin binding

C. albicans cells growing under iron limitation, as described above, were washed and enumerated. Approximately 5×10^5 cells were added per well in a 24 well plate containing Poly-L-Lysine-coated 12-mm diameter glass cover slips and 1 ml RPMI 1640 with no additions, with 100 µg ferritin or with 100 µg apoferritin (ferritin without iron [Sigma-Aldrich]). The cells were incubated for 4 h at 37°C under 5% CO₂. Afterwards, the cells were washed four times with ultra pure water, removed from the coverslips using a pipette point and resuspended in 1 ml ultra pure water. Serial dilutions of the cell suspensions were spotted on SD agar (described bellow) buffered 25 mM HEPES (pH 7.4) with or without 350 µM BPS (iron chelator). All plates were incubated for four days at 37°C.

3.16. Immunofluorescence of infected epithelial cells

Ferritin-enriched epithelial cell monolayers (monolayers treated with iron, described in section 3.6) were washed twice with PBS and infected with $\sim 10^5$ *C. albicans* cells in serum-free RPMI 1640 medium for 6 h. Next, the samples were washed twice with PBS and fixed with 500 µl Roti[®]-Histofix 4%. *C. albicans* cells and TR146 cells were incubated separately and used as controls. All incubation times were performed in a humidified incubator at 37°C and 5% CO₂.

To stain *C. albicans* cells localized only outside of epithelial cells, samples were incubated with 12.5 µg Concanavalin A - fluorescein conjugate (Invitrogen) in PBS for 45 min at room temperature before permeabilization. After washing, the cells were permeabilized by incubation with 0.1% Triton X-100 for 15 min at room temperature. After washing three times with PBS, the samples were blocked using Image-iT[™] FX

signal enhancer (Invitrogen) for 30 min at room temperature in a humidity chamber. After washing three times with PBS, the cells were incubated with rabbit anti-ferritin antibody coupled with dye DY-649 diluted 1:1000 in PBS with 1% BSA for 1 h at room temperature. To stain *C. albicans* cells localized outside and inside epithelial cells, the samples were incubated with 10 µg/ml Calcofluor White (Sigma) in 0.1 M Tris-hydrochloride (pH 9.0 [Roth]) for 20 min at room temperature.

Additionally, for some experiments the cell membrane of the epithelial cells was stained using Cell TrackerTM CM-Dil (Invitrogen) following the manufacturer's instructions.

Finally, cover slips were washed three times with ultra pure water, inverted and mounted on a microscope slide with ProLong® Gold Antifade Reagent. At least two experiments in duplicates were analyzed using a Leica microscope and 10 randomly chosen fields per cover-slip were photographed. A representative picture of each strain was selected.

3.17. *C. albicans* transformation with CIp10

To maintain an isogenic background among the strains used for the microarray experiments (see below), the *URA3* gene was reconstituted in the *Δhgc1* and *Δras1* mutant strains using the plasmid CIp10 (Murad *et al.*, 2000) and the strain CAI4 carrying CIp10 was used as the wild type. The mutant strains were transformed with CIp10 using the improved transformation protocol for *C. albicans* (Walther and Wendland, 2003). To confirm integration of the plasmid at the RP10 locus, colony-PCR was performed using primers RPF-1 (5'-gagcagtgtacacacacacatcttg-3') and URA-F2 (5'-ggagtggattagatgataaaggatgatgg-3').

3.18. Sample preparation for RNA extraction

C. albicans cells growing under iron limitation, as described above, were washed and enumerated. Approximately 2×10^6 cells per well were added in a 24 well plate containing Poly-L-Lysine-coated 12-mm diameter glass cover-slips in 1ml RPMI 1640 with 100 µg/ml ferritin. After 1.5 h incubation at 37°C and 5% CO₂, the medium was removed and 100 µl peqGOLD RNAPure (PeqLab) was added per well. The cells were immediately removed from the cover-slips using a pipette point. For each strain, cells from 12 wells were pooled in a microcentrifuge tube and immediately shock frozen in

liquid nitrogen. To verify that ferritin was bound to the *C. albicans* hyphae, additional cover slips for each strain were fixed and ferritin was stained as described above.

3.19. RNA extraction and labelling

Frozen cells were lysed and homogenized (Precellys 24, PeqLab) with glass beads (0.5 mm, Roth). Total RNA was extracted as previously described (Fradin *et al.*, 2005). Total RNA was linearly amplified and labeled using the 'Low RNA Input Fluorescent Linear amplification Kit' (Agilent Technologies).

3.20. Microarray hybridization and analysis

For transcriptional profiling, *C. albicans* microarrays (Eurogentec) were used as previously described (Fradin *et al.*, 2005). RNA was co-hybridized with a common reference (RNA from SC5314 grown in YPD, mid-log phase, 37°C). Slides were hybridized, washed and scanned as described (Fradin *et al.*, 2005). Data normalization (LOWESS) and analysis were performed in Gene-Spring 7.2 software (Agilent Technologies). Reliable expression of genes was defined as normalized expression of present genes that did not vary more than 1.5 standard deviations within replicate arrays. Genes were defined as differentially expressed if their expression was at least 2 times stronger or 2 times weaker in at least one strain compared to the common reference. Using the Benjamini and Hochberg false discovery test, a *p*-value < 0.05 was considered as significant. Microarray data from four independent experiments (two of them with dye swap) were used. To identify genes involved in ferritin binding, genes were selected that were up-regulated (≥ 2.5 stronger expression compared to common reference) in wild-type and $\Delta hgc1$ cells, but unaltered or down-regulated (≤ 1.5 of the common reference expression) in the $\Delta ras1$ mutant.

Raw data have been deposited in NCBI's Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through GEO series accession number GSE11490.

4. Results

4.1. Investigation of the influence of iron limitation on *C. albicans* growth

4.1.1. Iron limitation inhibits the growth of *C. albicans* in its yeast form

Iron is essential for almost all microbes. However, the amount of iron required for growth is species-dependent. To study the influence of iron limitation on yeast growth of *C. albicans*, a limited iron medium (LIM) was used. Because this medium is buffered to pH 4.2, it promotes yeast growth at 37°C. Therefore, more than 96% of the cells in all conditions were yeast cells (Table 4.1).

For growth analysis, cells from a YPD preculture were inoculated into LIM media with or without iron addition. Under these conditions, addition of 20 µM ferric iron (LIM20) was necessary to obtain exponential growth, resulting in an optical density (630 nm, OD₆₃₀) of 3.83 after 15 h incubation (Figure 4.1). The generation time in the log phase of cells growing in LIM20 was 79 min. The addition of 1 µM ferric iron (LIM1) allowed growth, but to lesser extent than 20 µM ferric iron, with a generation time in the log phase of 96 min. After 15 h incubation the OD₆₃₀ achieved by cells growing in LIM1 was only 1.52 (Figure 4.1). In contrast, without addition of iron (LIM0), no growth was observed (Figure 4.1). Thus, under these conditions, 20 µM ferric iron was required for optimal growth in the yeast form.

Table 4.1. Yeast and hyphal forms in LIM medium

	Yeast form (%)	Hyphal form (%)
LIM0 (no iron addition)	96.02	3.98
LIM1 (addition of 1 µM ferric iron)	97.61	2.39
LIM20 (addition of 20 µM ferric iron)	98.43	1.57

At least 100 cells per sample were counted.

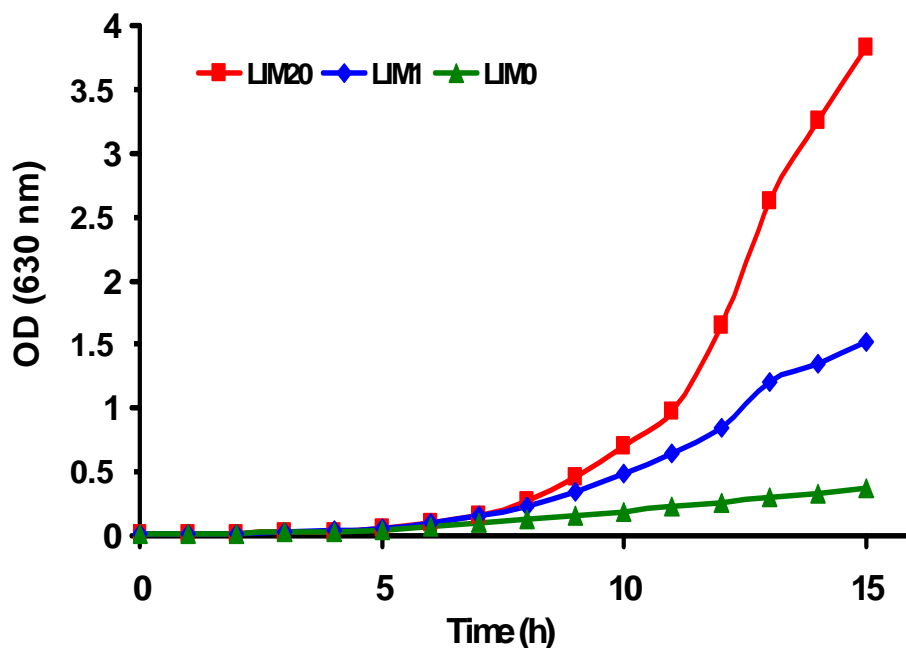


Figure 4.1. Iron limitation inhibits *C. albicans* growth

C. albicans (SC5314) cells from YPD preculture were washed and inoculated in LIM medium without addition of iron (LIM0), with 1 μ M ferric iron (LIM1) or with 20 μ M ferric iron (LIM20). The cells were grown in a rotation incubator at 37°C and 150 rpm and the optical density (630 nm) was measured every hour during 15 hours. While optimal growth was observed with 20 μ M ferric iron, growth was reduced with 1 μ M ferric iron and strongly inhibited without any iron.

4.1.2. Iron limitation inhibits the hyphal growth form of *C. albicans*

As mentioned above, the low pH of LIM medium induces yeast rather than hyphal growth. Hence, to analyse hyphal growth under iron limitation, cells were inoculated in a medium which induced hyphal formation (RPMI medium, pH 7.2) with an iron chelator (25 μ g/ml apotransferrin). Formation and length of hyphae were investigated microscopically. From an inoculum with yeast cells, *C. albicans* grew as hyphae in RPMI medium (87.17% hyphal cells after 12 h) (Figure 4.2A). The addition of apotransferrin reduced hyphal formation by approximately one third (after 12 h, 54.79% of the cells were hyphae) in comparison to RPMI without apotransferrin and this inhibition was reversed by the addition of 100 μ M ferric iron (Figure 4.2A).

In RPMI medium the hyphal length increased over time, from 59.48 ± 10.50 μ m at 6 hours to 157.69 ± 18.96 μ m at 12 hours post inoculation (Figure 4.2B). In contrast, when apotransferrin was present in the medium, the hyphae grew to only

$10.12 \pm 1.23 \mu\text{m}$ by 6 hours and a significant increase in hyphal length during the entire incubation time was not observed ($11.95 \pm 1.64 \mu\text{m}$ at 12 hours) (Figure 4.2B).

The inhibition of hyphal elongation by iron chelation with apotransferrin was reconstituted to control levels (RPMI medium) when $100 \mu\text{M}$ ferric iron was added, and the addition of iron to RPMI showed no increase or decrease in hyphal formation and elongation (Figure 4.2). Representative pictures of hyphal elongation in RPMI and RPMI with apotransferrin are shown in Figure 4.3.

These results demonstrated that iron chelation with apotransferrin, results in reduced hyphal formation and inhibition of hyphal elongation.

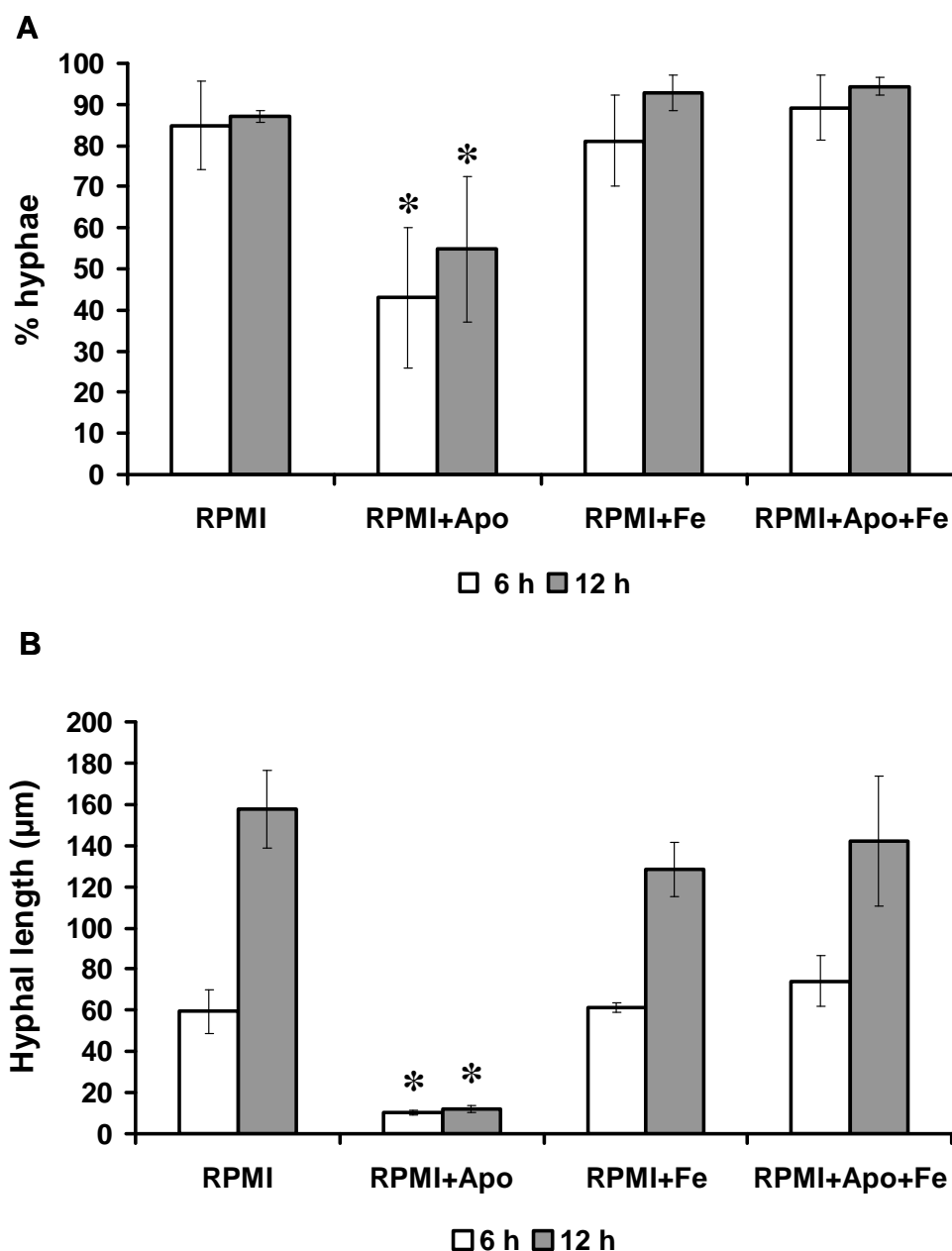


Figure 4.2. Iron is essential for hyphal elongation

The percentage of hyphae (**A**) and hyphal length (**B**) were determined after 6 h and 12 h of incubation with and without the natural iron chelator apotransferrin. Removal of iron inhibited hyphal extension. RPMI, RPMI medium with no additions; RPMI+Apo, RPMI medium with 25 μg/ml apotransferrin; RPMI+Fe, RPMI with 100 μM ferric iron; RPMI+Apo+Fe, RPMI medium with 25 μg/ml apotransferrin and 100 μM ferric iron. The cells were incubated at 37°C. The experiment was performed three times in duplicate. * indicates significant difference compared to cells growing in RPMI medium ($p < 0.05$).

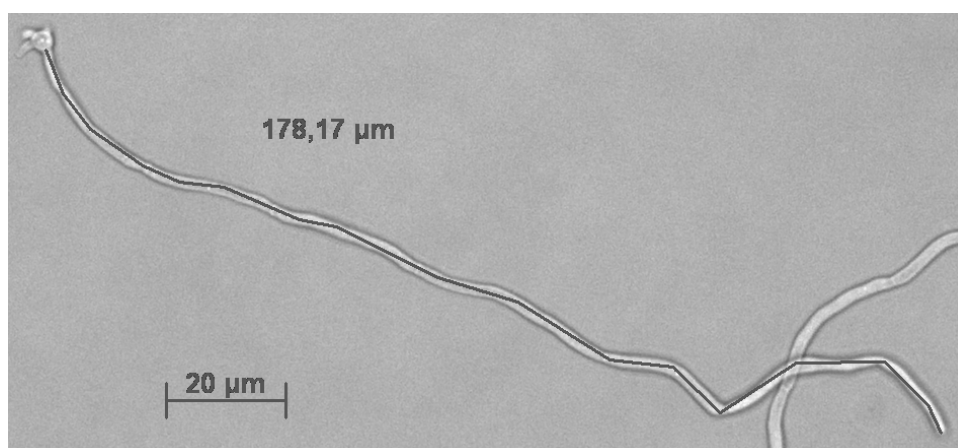
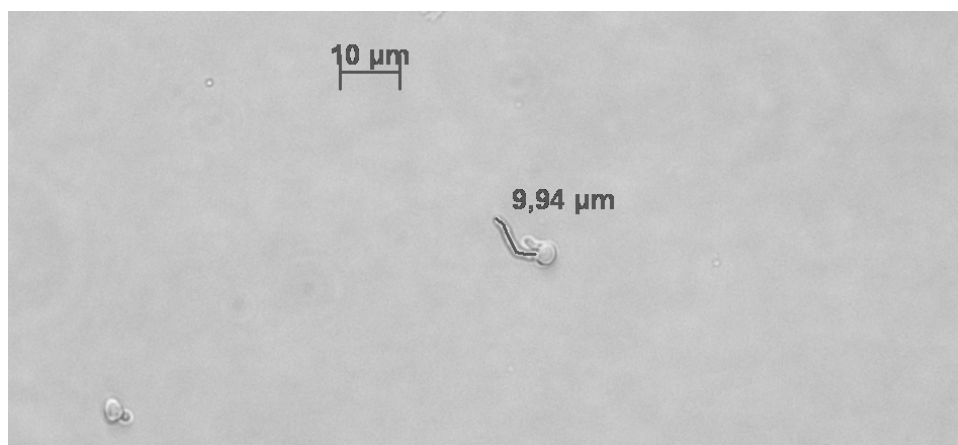
RPMI**RPMI + apotransferrin**

Figure 4.3. Inhibition of hyphal elongation through iron chelation by apotransferrin

C. albicans (SC5314) cells were grown for 12 h in RPMI medium with or without addition of 25 µg/ml apotransferrin and hyphal length was measured microscopically. The hyphal length was dramatically reduced in the presence of apotransferrin.

4.1.3 *C. albicans* can store iron intracellularly

Although the experiments above showed that lack of iron strongly inhibited proliferation, growth was not completely blocked. This suggested that *C. albicans* may possess an intracellular iron storage capacity, which can promote some cell divisions or germ tube formation under iron limitation.

To further investigate this possibility, two different iron chelators were used. Bipyridine is an iron chelator that can be internalised by the cells. Therefore, it is able to chelate

iron from the intracellular iron pool, removing intracellular iron resources. However, bathophenanthrolinedisulfonic acid disodium salt (BPS) is able to sequester only extracellular iron, so that intracellular iron resources are still available to the fungal cells.

C. albicans cells grown in Sabouraud medium had a generation time of 83.32 min in the log phase. The addition of 50 μ M bipyridine (intra- and extracellular iron chelator) increased the generation time to 129.12 min. Increasing the chelator concentration increased the inhibitory effect, with the addition of 200 μ M bipyridine abolishing growth (Figure 4.4A).

The addition of 50 μ M BPS (extracellular iron chelator) to Sabouraud medium also increased the generation time (126.34 min) similar to 50 μ M bipyridine (Figure 4.4A and B). In contrast to bipyridine, 200 μ M BPS did not abolish growth. Furthermore, even addition of 500 μ M or 1 mM BPS did not completely abolish growth (Figure 4.4B).

These results demonstrated that *C. albicans* cell division was completely inhibited by iron chelation, only when all intracellular iron was removed using bipyridine.

The presence or absence of an iron reservoir could influence the reproducibility of growth under iron limited conditions or virulence. Thus, in further experiments, iron starved cells (preculture in LIM0) were used.

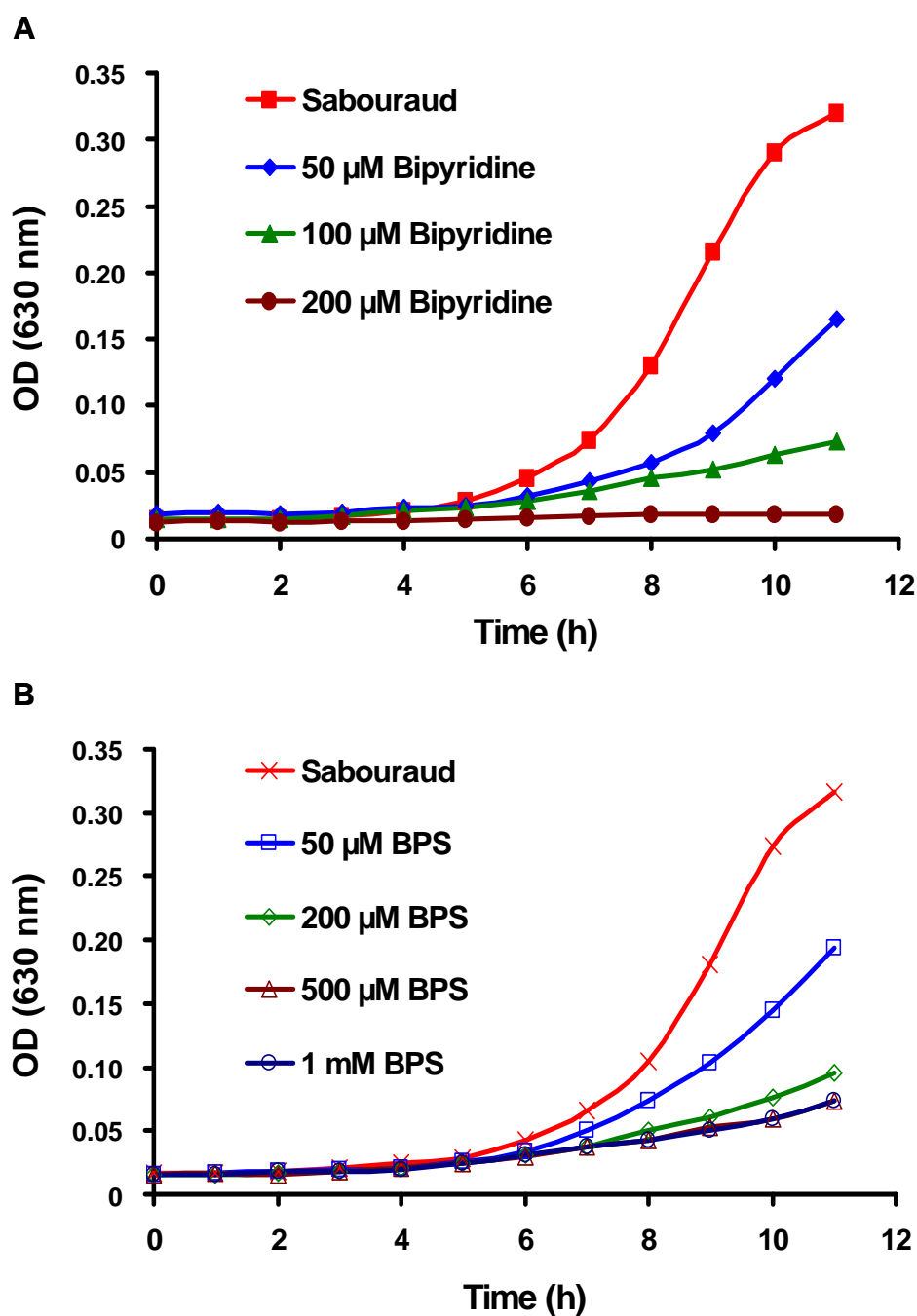


Figure 4.4. Growth inhibition by two different iron chelators

A, cells from YPD preculture were washed and inoculated into Sabouraud medium with increasing bipyridine concentrations (0 to 200 µM). **B**; cells from YPD preculture were washed and inoculated into Sabouraud medium with increasing BPS concentrations (0 to 1 mM). The *C. albicans* (SC5314) cells were incubated at 37°C while shaking at 150 rpm. The optical density (630 nm) was measured every hour. Inhibition of growth by both compounds was dose-dependent.

4.2. Analysis of *C. albicans* growth with ferritin as the sole source of iron

4.2.1. The ferritin content of epithelial cells influences the extent of cellular damage caused by *C. albicans*

As previously shown, *C. albicans* needs iron to proliferate. Once the fungus has invaded and damaged oral epithelial cells, it is expected that *C. albicans* can exploit iron sources from those cells.

To determine how the availability of iron influences fungal-host cell interactions, oral epithelial cell monolayers were incubated in the presence of additional free iron or the iron chelator BPS. Through immunocytochemical localization of ferritin within epithelial cells, it was found that exclusion of fetal bovine serum (FBS) and addition of BPS caused a dramatic decrease in cellular ferritin within 24 hours of incubation (Figure 4.5A) compared to control cells (incubated in RPMI with 10% FBS and without BPS) (Figure 4.5B). In contrast, addition of free iron to the medium (RPMI with 10% FBS and 50 μ M iron chloride) increased the concentration of ferritin within the same time period (Figure 4.5C). The treatment of epithelial cells with additional iron or with the iron chelator alone did not cause cell damage, as monitored by measuring the release of epithelial lactate dehydrogenase (LDH) into the supernatant.

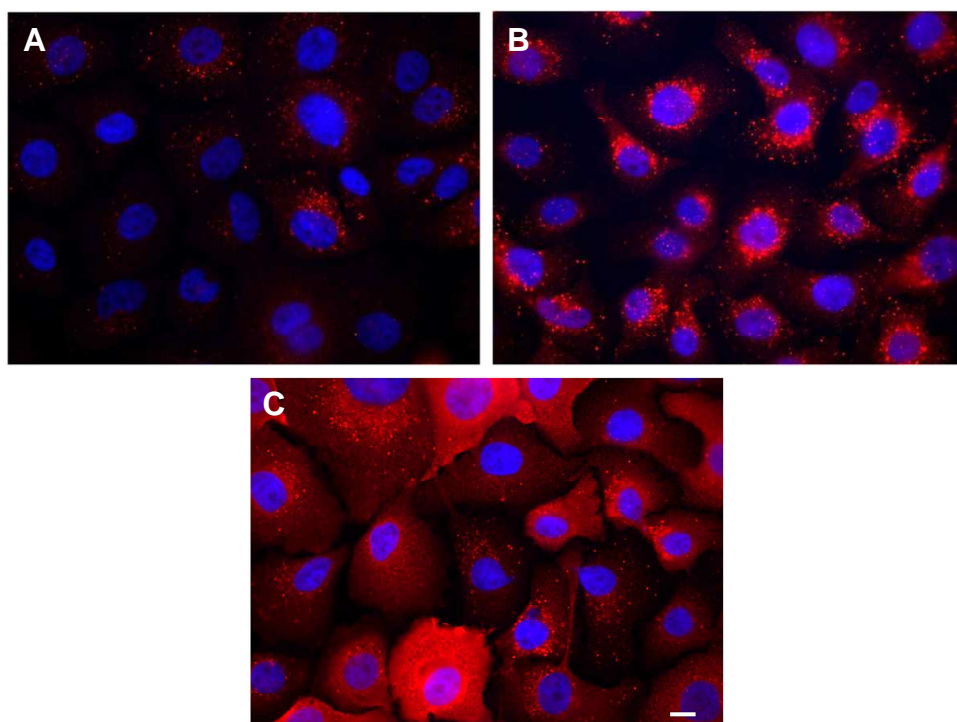


Figure 4.5. The ferritin content of host epithelial cells is iron regulated

The ferritin content of cells was monitored using immunofluorescence. In blue, nuclei stained with DAPI. In red, immunofluorescent staining using anti-ferritin antibody. **A**, monolayer incubated for 24 h in serum-free RPMI with 50 μ M BPS. **B**, monolayer incubated for 24 h in RPMI with 10% FBS (control). **C**, monolayer incubated for 24 h in RPMI with 10% FBS and 50 μ M iron chloride. The ferritin content increased from **A** to **C**. Bar indicates 10 μ m. The same protocol and exposure times were used for all three conditions. The experiment was performed at least twice in duplicate.

Next, the treated epithelial monolayers (ferritin enriched or depleted monolayers) were incubated for 8 h with *C. albicans* in normal cell culture medium (serum-free RPMI) and cell damage caused by *C. albicans* was monitored by measuring LDH release.

The epithelial monolayers treated with iron chelator (depleted of ferritin) displayed significantly less LDH release after incubation with the fungus in comparison to control cells (Figure 4.6). In contrast, ferritin enriched epithelial cells suffered more damage by *C. albicans* and this increase in damage was dose dependent (Figure 4.6). This observation suggested that the ferritin content of epithelial cells directly correlates with cell damage and opened up the possibility that *C. albicans* can use ferritin as an iron source.

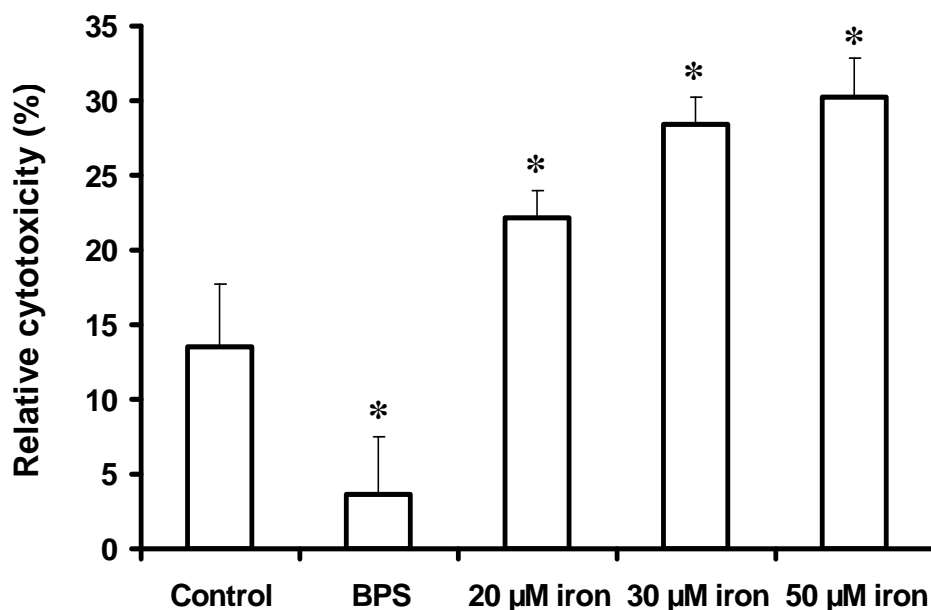


Figure 4.6. The ferritin content of host epithelial cells influences cell damage by *C. albicans*

Following the treatments described in Figure 4.5, the monolayers were washed and incubated for 8 h in serum-free RPMI with 10^6 *C. albicans* (SC5314) cells. Cell damage was quantified by monitoring the release of epithelial LDH into the medium and calculated in relative cytotoxicity (%). Control, monolayers preincubated in normal cell culture medium (RPMI with 10% FBS); BPS, monolayers preincubated in serum-free RPMI with 50 µM BPS (iron chelator); 20, 30 and 50 µM iron, monolayers preincubated in cell culture medium with 20, 30 and 50 µM ferric iron respectively. Increasing ferritin concentrations directly correlated with increasing cytotoxicity of *C. albicans*. The experiments were performed twice in duplicate. * indicates significant difference compared to the control ($p < 0.05$).

4.2.2. Development of a method to purify ferritin from free iron contamination

Since the ferritin content of epithelial cells correlated with cell damage, it was postulated that *C. albicans* may be able to use ferritin as an iron source. To investigate whether the fungus can use ferritin as an iron source for growth under *in vitro* conditions (see 4.2.3), a commercial solution of horse spleen holoferitin (ferritin charged with iron) was used. To exclude the possibility that free iron contamination in the ferritin solution may support fungal growth, the free iron content of the solution was analysed.

First, a method to quantify iron was developed. Guanidinium thiocyanate binds iron, generating a red colour in the solution. Using a spectrophotometer, the colour reaction using a standard solution of ferric iron was monitored and a standard curve generated. The R^2 value demonstrated that the method is reliable (Figure 4.7A).

Next, the ferritin solution (holoferritin from horse spleen) was diluted 1:10 in dilution buffer (5 mM HEPES; 0.1 M NaCl; pH 7.4) and passed through a Microcon YM-100 Centrifugal Filter Unit (cut off size 100 kDa). The retentate was collected in a new 1.5 ml microcentrifuge-tube. The dilution buffer was used to adjust the retentate to the original volume and the solution was again passed through a new column. This procedure was repeated 4 times. The eluate was used for iron quantification. The first eluate (first passage) contained $8.23 \pm 2.01 \mu\text{M}$ iron. The free iron concentration in the second eluate decreased to $2.87 \pm 0.54 \mu\text{M}$ iron (Figure 4.7B). Because there was no significant decrease in iron contamination in passages 3, 4 or 5 (compared to each other), the ferritin solution was washed once (as described above) before it was used in further experiments.

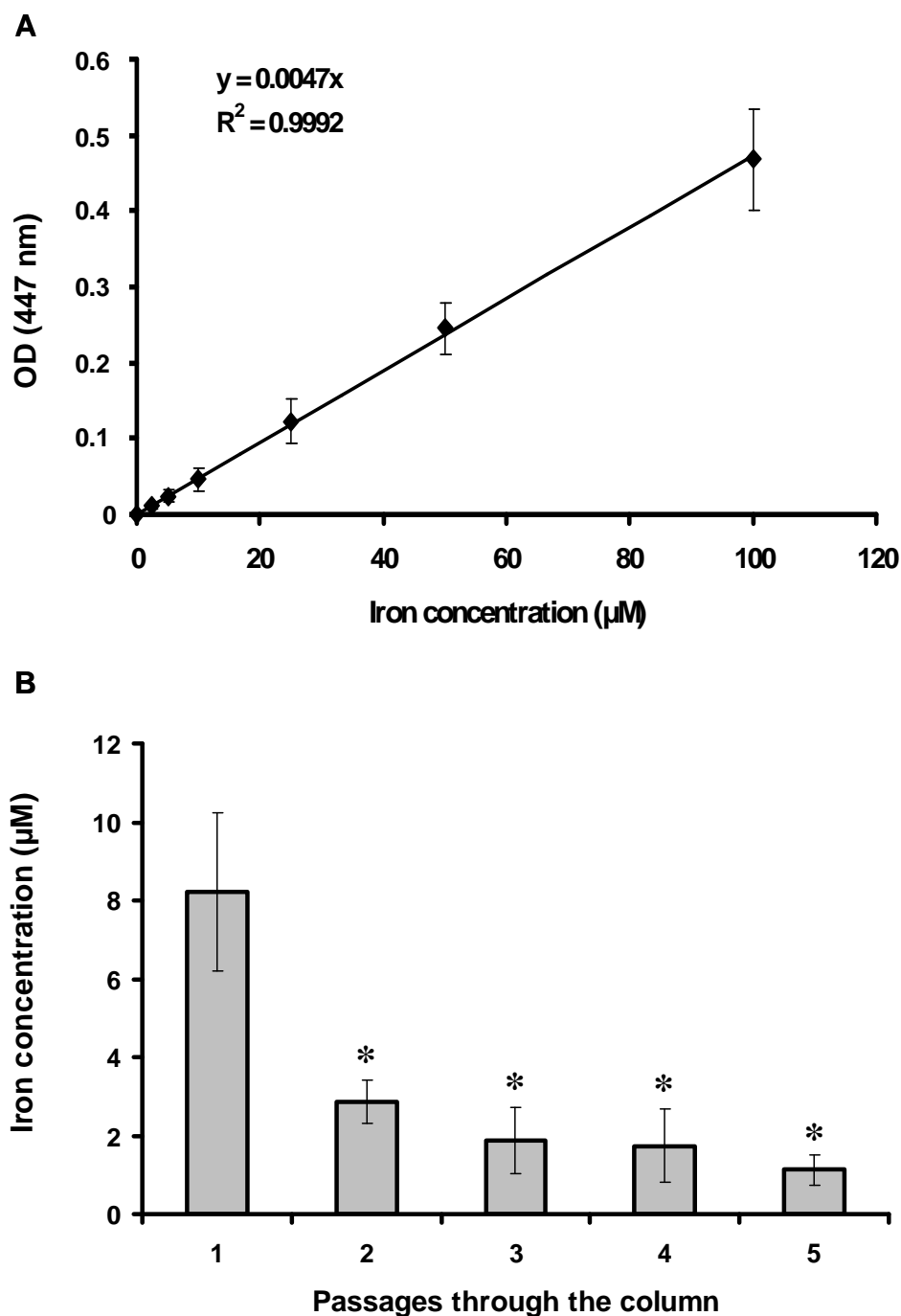


Figure 4.7. Free iron contamination in a commercial solution of ferritin

A, standard curve for iron quantification. A solution consisting of 1 M guanidinium thiocyanate diluted in 1 M HCl was mixed with increasing concentrations of ferric iron (2.5 μM, 5 μM, 10 μM, 25 μM, 50 μM and 100 μM) and absorbance was measured at 447 nm. The graph represents an average of three experiments performed in triplicate. The line equation and R^2 value are shown in the upper left corner. **B**, iron concentration (given in μM) in the eluate of a commercial horse spleen holoferritin solution after subsequent passages through a column (cut off size 100 kDa). A single passage through the column removed most of the iron contamination. * indicates significant difference compared to the first passage ($p < 0.001$).

4.2.3. *C. albicans* can use ferritin as the sole source of iron *in vitro*

Since the ferritin content of epithelial cells influenced cell damage caused by *C. albicans*, the fungus may be able to use ferritin as an iron source during infection of these cells. To investigate this hypothesis, *C. albicans* wild type cells were tested for growth on agar with ferritin as the sole iron source. The addition of 350 μ M BPS to the medium removed remaining iron contamination, blocking growth and the appearance of any colonies unless an external iron source was added. For example, addition of free ferrous iron, haemoglobin or ferritin promoted the growth of *C. albicans* at pH 7.4 (Figure 4.8). In contrast, the baker's yeast *Saccharomyces cerevisiae*, known to be unable to grow with haemoglobin as the sole source of iron, only grew with the addition of free iron to the medium (Figure 4.8).

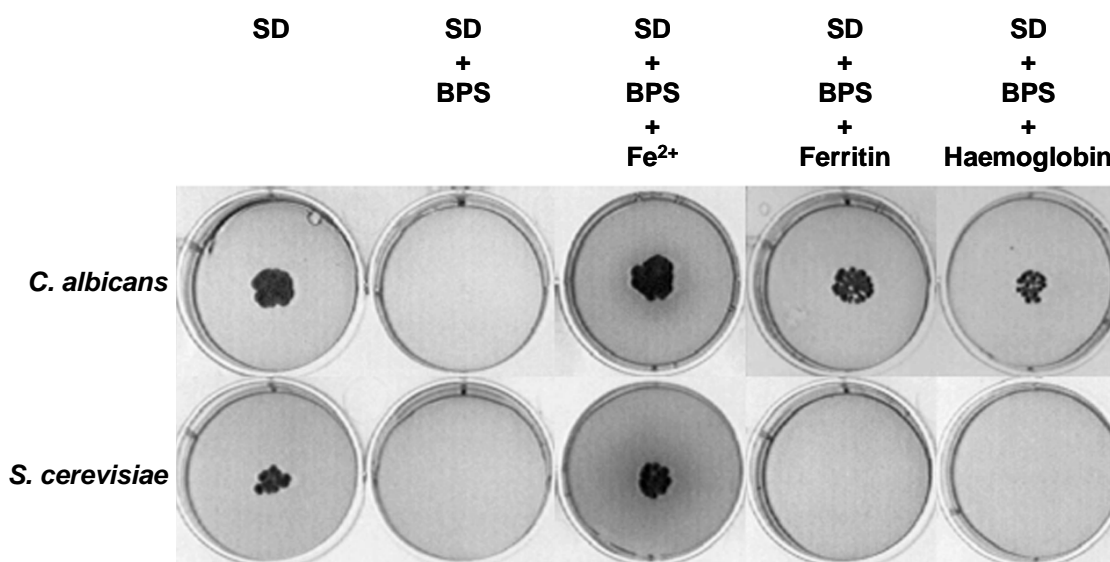


Figure 4.8. Ferritin: an iron source used by *C. albicans*

Colony growth of *C. albicans* and *S. cerevisiae* on SD agar plates with an iron chelator (BPS) and buffered with 25 mM HEPES at pH 7.4. Approximately 50 cells of *C. albicans* (SC5314) or *S. cerevisiae* (ATCC9763) were spotted on agar with the pH adjusted to 7.4 and incubated for 2 days at 37°C under 5% CO₂ (*C. albicans*) or 30°C (*S. cerevisiae*). Only *C. albicans* can grow with ferritin or haemoglobin as the sole iron source. Fe²⁺, 50 μ M iron sulphate; Ferritin, 20 μ g/ml holo-ferritin; Haemoglobin, 20 μ g/ml haemoglobin.

To investigate the effect of pH on ferritin utilisation, media were buffered to either neutral/alkaline or to acidic pH. As shown in Figure 4.9, *C. albicans* was able to grow with ferritin as the sole source of iron at pH 7.4 and 5.0. As described above, *S. cerevisiae* was not able to grow with ferritin as the sole source of iron at pH 7.4. Surprisingly, *S. cerevisiae* was able to grow with ferritin, when the initial pH of the medium was reduced to 5.0 (Figure 4.9). This result suggested that acidic pH could influences iron release from ferritin.

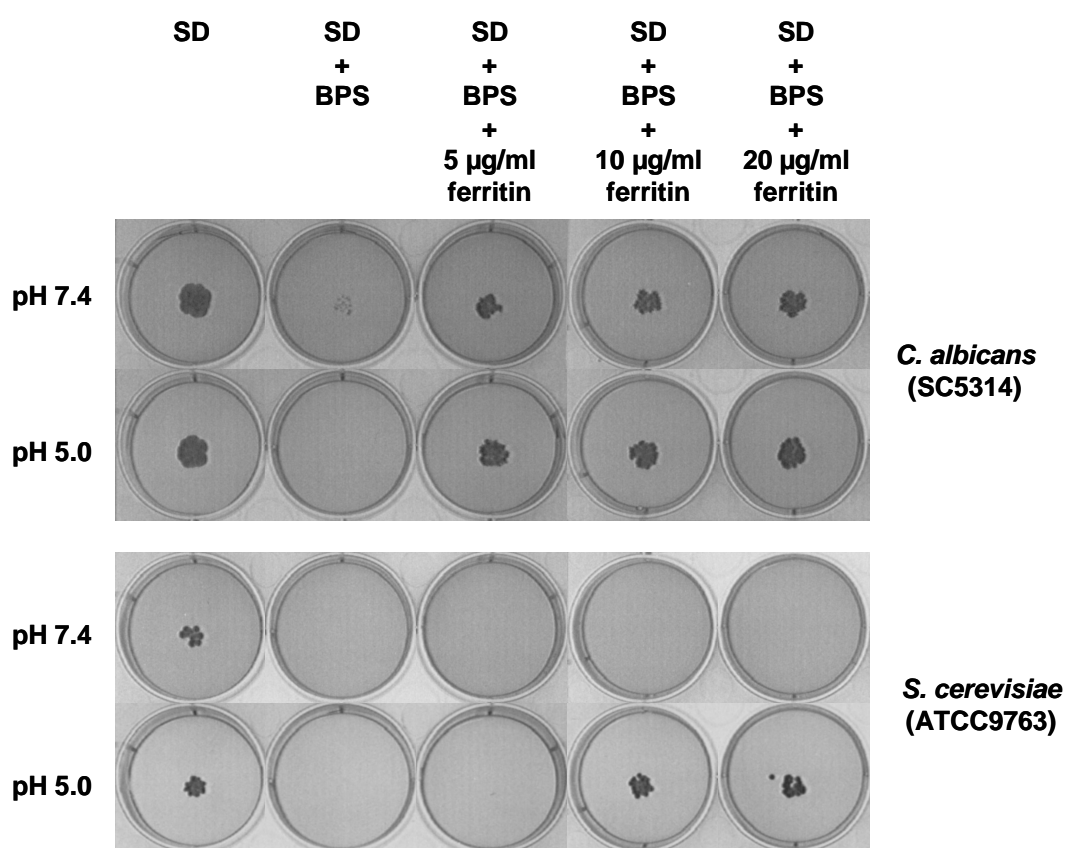


Figure 4.9. *S. cerevisiae* can grow on ferritin plates under acidic conditions

Colony growth on SD agar plates with an iron chelator (BPS) and buffered with 25 mM HEPES. Approximately 50 cells of *C. albicans* or *S. cerevisiae* were spotted on agar (pH 7.4 or 5.0) and incubated for 2 days at 37°C under 5% CO₂ (*C. albicans*) or at 30°C (*S. cerevisiae*).

4.2.4. Usage of ferritin as the sole source of iron *in vitro* requires acidification of the medium

The observation that the pH has an influence on the usage of ferritin as an iron source opened up the possibility that the fungi actively modify the surrounding pH of the medium. To investigate whether *C. albicans* and *S. cerevisiae* are able to acidify the surrounding medium during growth on agar, a pH indicator (bromocresol green) was added to the medium. Figure 4.10 shows that both *C. albicans* and *S. cerevisiae* were able to acidify the surrounding medium to values below pH 4.0 during growth on agar plates.

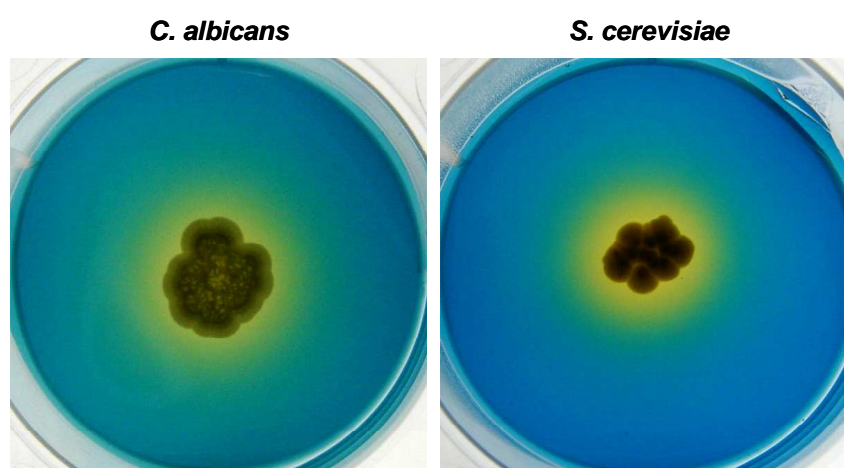


Figure 4.10. *C. albicans* and *S. cerevisiae* can acidify the surrounding medium

Colony growth of *C. albicans* and *S. cerevisiae* on agar and subsequent acidification of the surrounding medium indicates fungal acid production. Approximately 50 cells of *C. albicans* wild type (SC5314) or *S. cerevisiae* (ATCC9763) were spotted on SD agar with the pH adjusted to 7.4. The pH indicator bromocresol green was added to monitor pH changes. The plates were incubated for 4 days at 37°C under 5% CO₂ (*C. albicans*) or 30°C (*S. cerevisiae*). Blue staining indicates a pH above 6.0 and yellow staining indicates a pH below 4.0.

Because the ferritin protein shell is known to be unstable at acidic pH and both species, *S. cerevisiae* and *C. albicans*, were able to grow with ferritin at acidic pH, it is probable that *C. albicans* needs to acidify the medium to release iron from this protein. To test this hypothesis, the carbon source of the medium was changed from glucose to casamino acids. This mixture of amino acids can be used as a carbon source, avoiding glucose-induced acidification (Lapathitis and Kotyk, 1998; Kotyk *et al.*, 1999). Additionally, the buffering capacity of the medium was increased from 25 to 200 mM HEPES. These changes in the media composition decrease the acidification of the

media by *C. albicans*. As showed in figure 4.11, decreasing the capacity of the fungus to acidify the medium also decreased its ability to grow with ferritin.

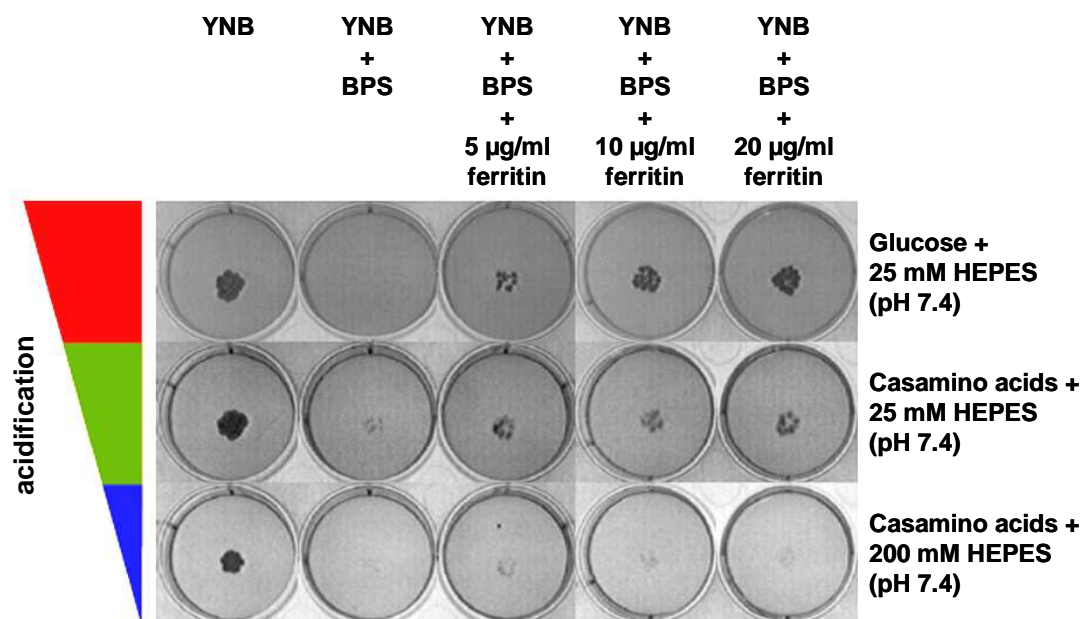


Figure 4.11. The effect of media acidification on growth with ferritin

Growth on agar plates with an iron chelator (BPS) and increasing concentrations of ferritin under conditions which favour (red) or which prevent (blue) acidification. Approximately 50 cells of *C. albicans* (SC5314) were spotted on YNB agar supplemented with either glucose (SD medium) or casamino acids as carbon sources and buffered with 25 or 200 mM HEPES. The initial pH of the media was 7.4.

4.2.5. Functional mutant screening reveals that the reductive pathway is essential for iron acquisition from ferritin

To investigate the genetic basis of iron acquisition from ferritin, mutants with defects in the three known high-affinity iron uptake systems of *C. albicans* were investigated. Mutants lacking key genes of each iron acquisition system were screened for growth on ferritin agar plates. The mutants lacking the siderophore transporter Sit1 ($\Delta sit1$) and the haemoglobin receptor Rbt5 ($\Delta rbt5$) were able to grow with ferritin as the sole source of iron (Figure 4.12). In contrast, mutants lacking the iron permease Ftr1 ($\Delta ftr1$) and the copper transporter Ccc2 ($\Delta ccc2$), both components of the reductive pathway, lost their abilities to grow with ferritin as the sole iron source (Figure 4.12). Reconstitution of one copy of the relevant gene in those defective mutants recovered growth on ferritin plates.

One further activity which may contribute to iron acquisition from ferritin is proteolysis. Thus, the possibility existed that the well-investigated aspartic proteases from *C. albicans* are involved in ferritin degradation and iron release. The aspartic protease triple mutants $\Delta sap1-3$ and $\Delta sap4-6$ were therefore tested for their abilities to acquire iron from ferritin. As these mutants displayed the wild type phenotype on ferritin plates (Figure 4.12), it seems that the secreted aspartic proteases Sap1-6 are not involved in this process.

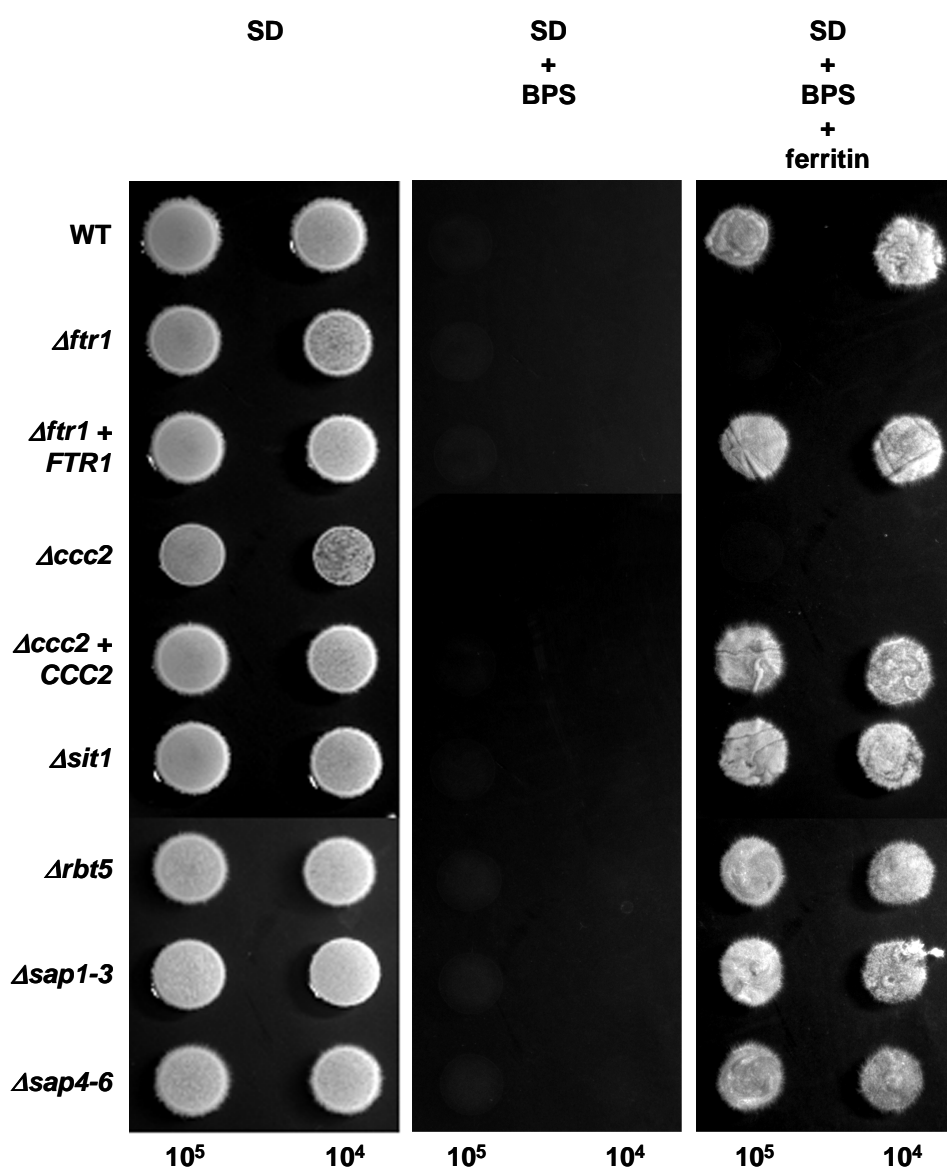


Figure 4.12. Components of the reductive pathway are required for growth on ferritin agar plates
SD agar was buffered using 100 mM HEPES (pH 7.4). BPS, iron chelator; Ferritin, 5 μ g/ml ferritin. Cells from *C. albicans* mutants lacking key genes of the three iron uptake systems or protease genes were spotted at two concentrations (left to right, 10^5 and 10^4 cells, respectively) for each strain. Revertant strains were tested for those mutants which displayed a growth defect. All plates were incubated for 3 days at 37°C under 5% CO₂.

Furthermore, *Δftr1* and *Δccc2* mutants were unable to grow on ferritin plates with an initial pH of 5.0 (Figure 4.13), demonstrating a total reliance on the reductive pathway for iron acquisition from ferritin.

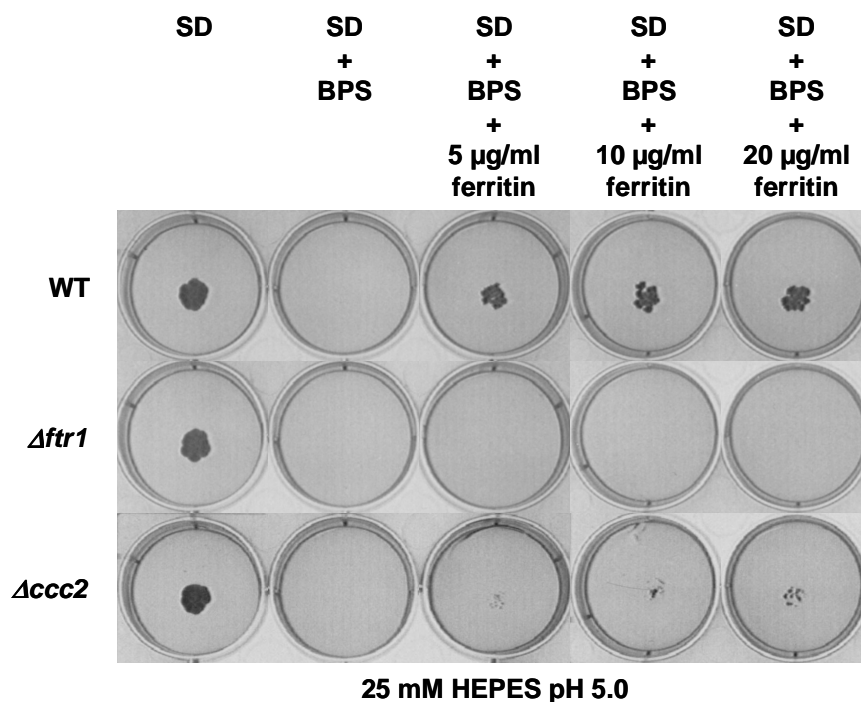


Figure 4.13. Acidic conditions do not permit growth of *Δftr1* or *Δccc2* on ferritin agar plates

Growth on agar plates with an iron chelator (BPS) and increasing concentrations of ferritin. *C. albicans* wild type (SC5314), *Δftr1* and *Δccc2* cells were spotted on agar with pH adjusted to 5.0. All plates were incubated for 2 days at 37°C under 5% CO₂. Neither *Δftr1* nor *Δccc2* cells were able to grow under these conditions.

4.2.6. Iron uptake from haemoglobin is independent from the reductive pathway

As described in section 4.2.3, *C. albicans* was able to grow with haemoglobin and ferritin as the sole iron source. Furthermore, deletion of components of the reductive pathway precluded growth with ferritin. However, the mutant lacking the iron permease gene *FTR1* was able to grow with ferrous iron sulphate and with haemoglobin as iron sources (Figure 4.14). Therefore, utilisation of iron from haemoglobin is independent from the reductive pathway and it may be indirectly concluded that iron acquisition from ferritin is independent of the haemoglobin uptake system.

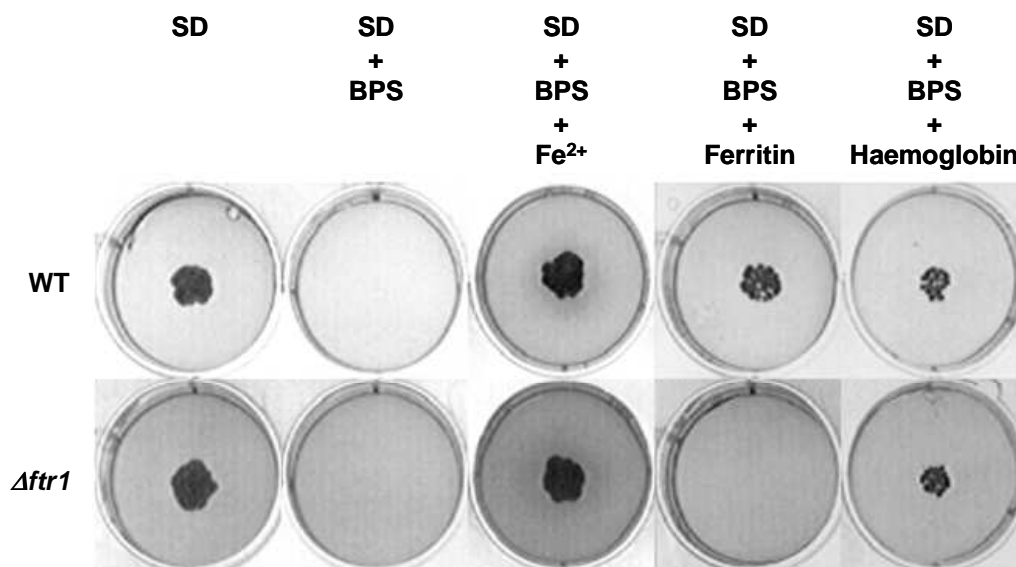


Figure 4.14. Iron uptake from haemoglobin is independent from the reductive pathway

Wild type and $\Delta ftr1$ cells were spotted on agar with the pH adjusted to 7.4. Fe^{+2} , 50 μM iron sulphate; Ferritin, 20 $\mu\text{g/ml}$ holo ferritin; Haemoglobin, 20 $\mu\text{g/ml}$ haemoglobin. All plates were incubated for 2 days at 37°C under 5% CO_2 . Growth of the $\Delta ftr1$ mutant is not reduced with haemoglobin as the sole source of iron.

4.2.7. The pH sensing pathway is involved in iron acquisition from ferritin

The above described experiments suggest that pH plays a key role in the utilisation of iron from ferritin. The *RIM101* pathway is responsible for pH sensing in *C. albicans* and defects in sensing of the environmental pH prevent an appropriate cellular response (Thewes *et al.*, 2007). Therefore, it may be possible that the induction of the iron reductive pathway under alkaline pH is blocked in mutants with a defective *RIM101* pathway. Thus, two mutants lacking genes from this pathway ($\Delta rim101$ and $\Delta dfp16$) were analysed for their ability to grow on ferritin plates. Both $\Delta rim101$ and $\Delta dfp16$ displayed iron uptake defects under alkaline pH (Bensen *et al.*, 2004; Thewes *et al.*, 2007). As expected, these mutants also displayed growth defects on ferritin plates at pH 7.4 but not at pH 5.0 where both $\Delta rim101$ and $\Delta dfp16$ cells grew at wild type rates on ferritin plates (Figure 4.15). These results show that sensing of an environmental alkaline pH is essential for iron uptake from ferritin.

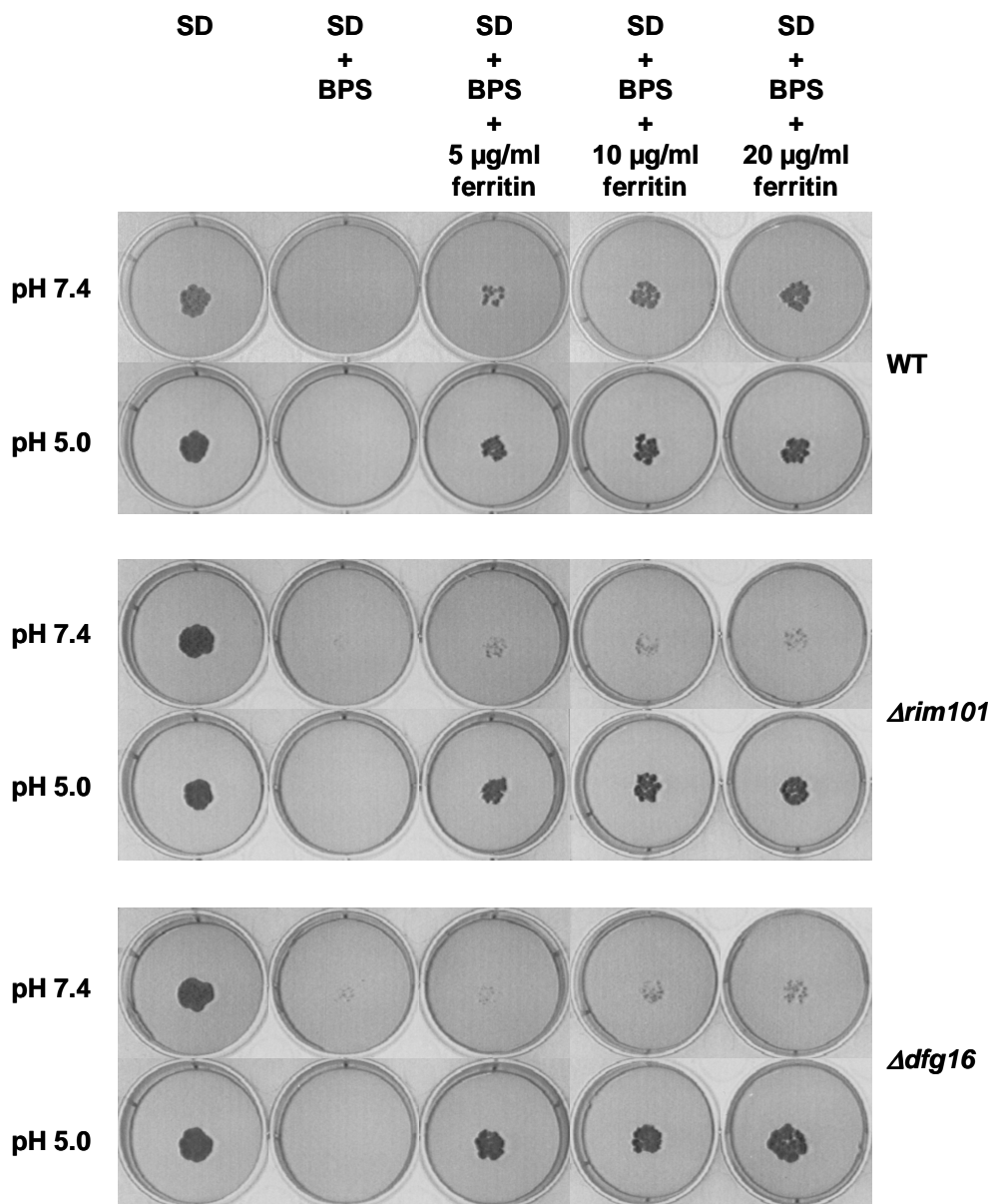


Figure 4.15. The *RIM101* pathway is involved in iron uptake from ferritin

Growth on SD agar plates with an iron chelator (350 μ M BPS) and increasing holoferritin concentrations. The medium was buffered with 25 mM HEPES and the pH adjusted as indicated. All plates were incubated for 2 days at 37°C under 5% CO₂. WT, *C. albicans* wild type (SC5314). The $\Delta rim101$ and $\Delta dfg16$ mutants, both lacking key genes of the *RIM101* pH sensing pathway, grew poorly at pH 7.5, but not at pH 5.0.

4.3. Properties of ferritin binding by *C. albicans*

4.3.1. Hyphal, but not yeast cells of *C. albicans* can bind ferritin

As described above, *S. cerevisiae* could not grow with ferritin as the sole source of iron when the initial pH was 7.4, although it was able to acidify the medium on SD agar plates with pH indicator. It is likely that *C. albicans* possesses mechanisms, such as ferritin binding, which enables close contact to the ferritin iron source and thus more efficient ferritin iron utilisation than *S. cerevisiae*.

To investigate whether *C. albicans* can bind ferritin, a ferritin-binding assay was performed. *C. albicans* wild type cells, precultured in LIM0 medium, were co-incubated with ferritin and ferritin molecules were subsequently visualized with fluorescent stained anti-ferritin antibodies. Almost 96% ($95.8\% \pm 1.53\%$) of all hyphae, but not yeast forms of *C. albicans* wild type cells displayed ferritin binding (Figure 4.16).

Since neither yeast cells, nor the mother-cells of hyphae displayed ferritin binding, the binding mechanism was proposed to be hyphal-specific. Therefore, mutants unable to form hyphae ($\Delta ras1$ and $\Delta cph1/efg1$) were tested for ferritin binding. $\Delta ras1$ and $\Delta cph1/efg1$ mutants did not produce any hyphae and were both completely unable to bind ferritin (Figure 4.16). To further analyse if ferritin binding was hyphal-specific, the $\Delta hgc1$ mutant was tested in the binding assay. This mutant cannot form true hyphae but can still express hyphal-specific genes (Zheng *et al.*, 2004). In fact, under hyphal-inducing conditions, $88.2\% \pm 6.84\%$ of all yeast or pseudohyphae cells of the $\Delta hgc1$ mutant were able to bind ferritin (Figure 4.16). These results suggested that genes expressed in true hyphal wild type cells or in yeast or pseudohyphae of the $\Delta hgc1$ mutant, but not in the $\Delta ras1$ and $\Delta cph1/efg1$ mutants, are essential for ferritin binding.

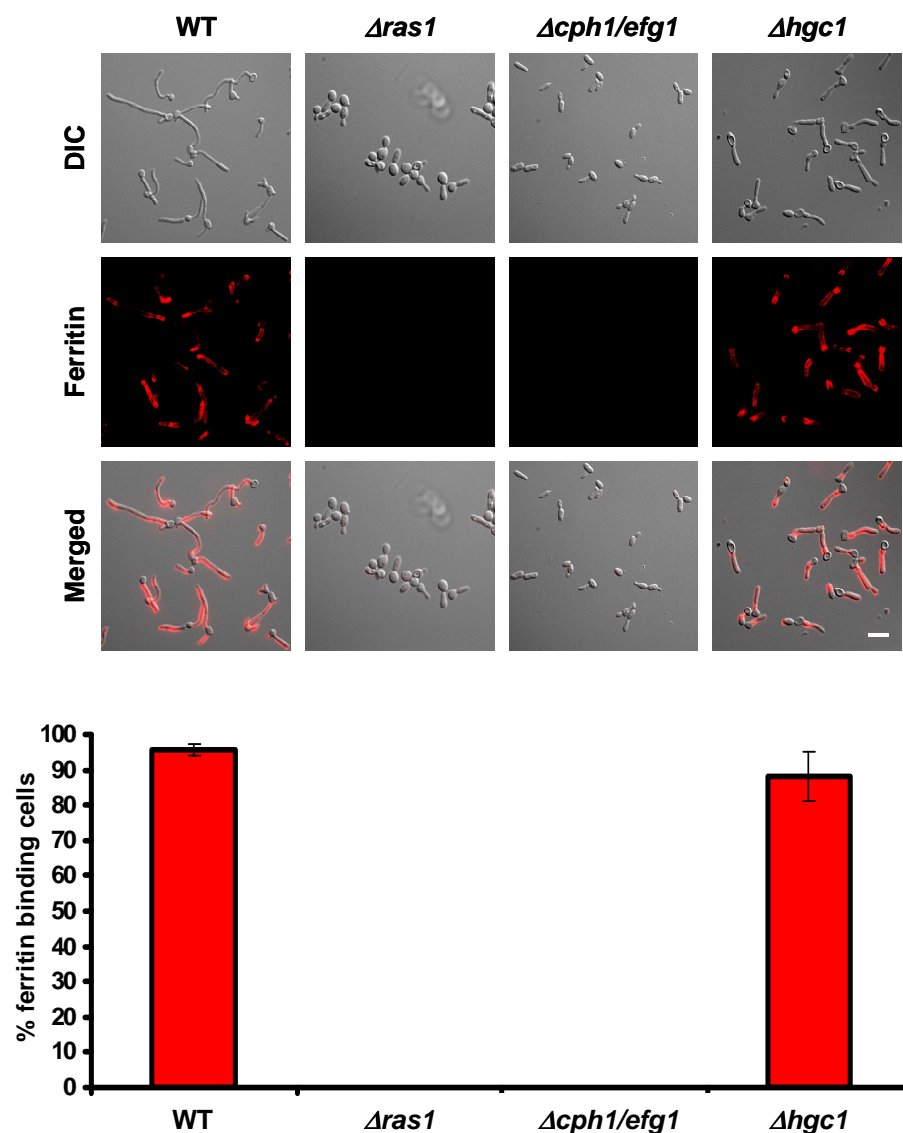


Figure 4.16. Ferritin binding of *C. albicans* requires hyphal formation

C. albicans wild type (SC5314) and mutant cells lacking key genes required for hyphal formation were incubated under hyphal-inducing conditions in the presence of 100 $\mu\text{g/ml}$ holo-ferritin. After 1 h, cells were washed and ferritin stained using immunofluorescence. Note that ferritin binding does not occur on yeast cells and on the mother cell of hyphae. DIC, **D**ifferential **I**nterference **C**ontrast. Bar indicates 10 μm . The graph shows the quantification of the cells binding ferritin. For each strain the % of cells displaying ferritin binding is given for > 100 randomly selected cells. The experiment was performed at least 3 times in duplicate.

4.3.2. Electron microscopy analysis of cells binding ferritin

The binding of ferritin on hyphal cells was visualized by electron microscopy. Due to their high electron density, ferritin molecules appeared as black particles in the transmission electron micrograph, close to the fungal cell wall, indicating that the binding of ferritin was on, but not within the fungal cell wall (Figure 4.17A). *C. albicans* cells incubated under the same condition, but without ferritin, had no such electron dense particles on their surfaces.

Electron spectroscopic imaging (a methodology for visualization of iron molecules) demonstrated that these dark particles were the ferritin iron core (Figure 4.17B).

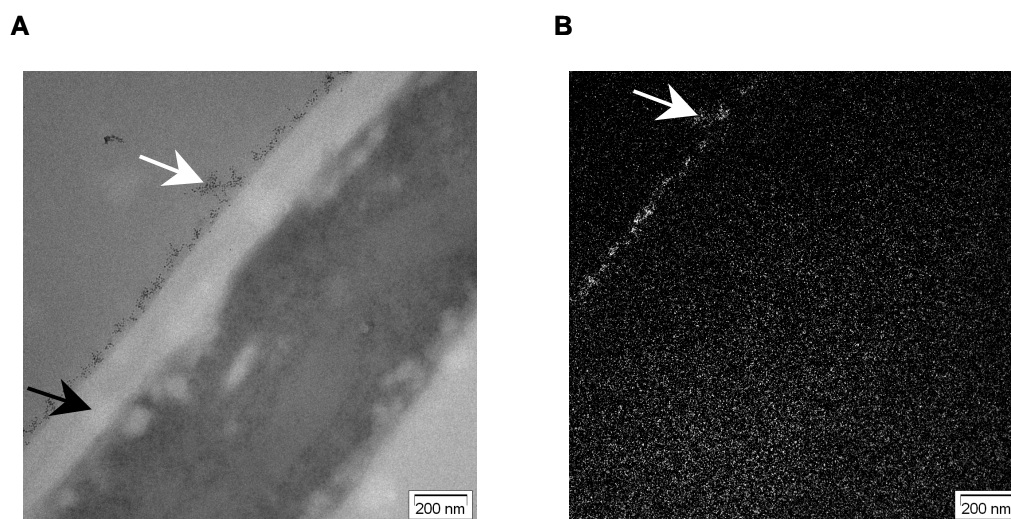


Figure 4.17. Ferritin binding is localized on the cell wall

A, wild type cells binding ferritin were analysed under transmission electron microscopy. The black arrow points to the cell wall, the white arrow to ferritin molecules visualized by their electron density.

B, electron spectroscopic imaging showing the localization of iron molecules. The white arrow points to the ferritin iron core. The white arrow points to the same position in panel **A** and **B**. Ferritin particles were localised on, but not within the cell wall.

4.3.3. Cell viability is not necessary for ferritin binding

To analyse if viability is necessary for *C. albicans* to bind to ferritin, two approaches were used. First, wild type hyphal cells were killed with thimerosal and tested for ferritin binding. As shown in Figure 4.18, thimerosal-killed cells were still able to bind ferritin ($89.04\% \pm 2.56\%$).

In the second approach, cells were killed using UV light and these cells completely lost their abilities to bind ferritin (Figure 4.18). When untreated wild type cells were mixed with 50% UV-killed cells, $49.06\% \pm 4.27\%$ of the cells bound ferritin.

These data suggested that hyphal formation, but not cell viability, is essential for ferritin binding. It is possible that exposure to UV light damaged the proteins on the cell surface and consequently the ferritin receptor.

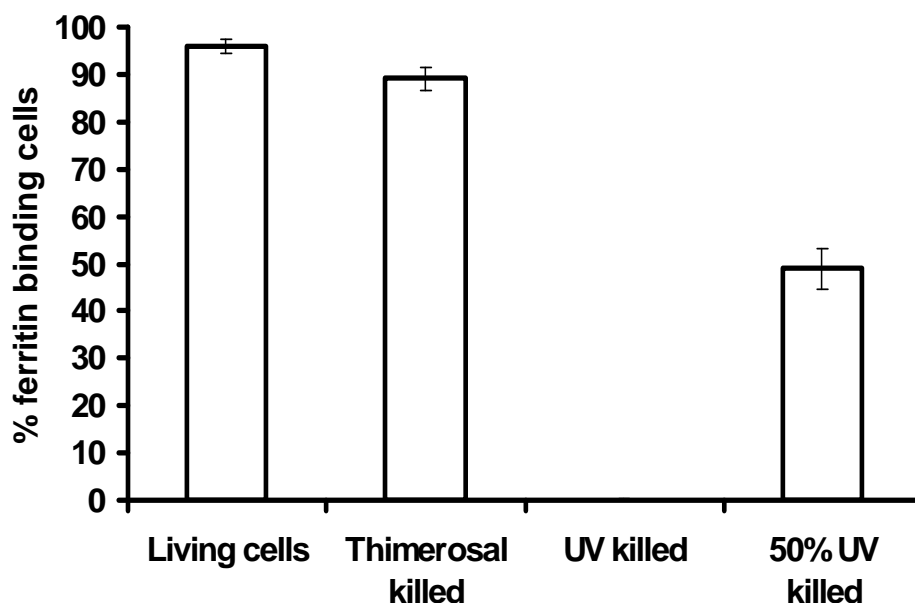


Figure 4.18. Ferritin binding requires an intact cell surface, but not viable cells

Comparison between living cells and dead cells (using thimerosal or UV light) in the ferritin-binding assay. The experiment was repeated at least twice performed in duplicate. Thimerosal killing did not reduce binding, while UV treatment blocked binding.

4.3.4. Ferritin binding is not iron regulated

To determine whether the ferritin iron core influences binding, *C. albicans* hyphae were incubated with holoferritin (iron charged ferritin) or apoferritin (ferritin shell without iron). The hyphal cells were able to bind holoferritin and apoferritin with similar efficiency (Figure 4.19A), indicating that iron molecules within the ferritin shell were dispensable for binding of ferritin.

To further analyse the role of iron in ferritin binding by *C. albicans*, cells from a LIM0 (iron starved cells) or YPD (cells with iron storage) preculture were compared in their abilities to bind ferritin. Almost 94% ($93.9\% \pm 5.3\%$) of the iron starved cells bound

ferritin. Similarly, around 95% ($94.9\% \pm 3.3\%$) of the cells from a YPD preculture bound ferritin. Finally, cells from a YPD preculture were incubated with ferritin in the presence of 50 μM ferric iron. Again, these cells showed no differences in ferritin binding compared to the other conditions (Figure 4.19B).

Thus, these data indicated that the binding of ferritin by *C. albicans* was morphology associated, but not iron-regulated.

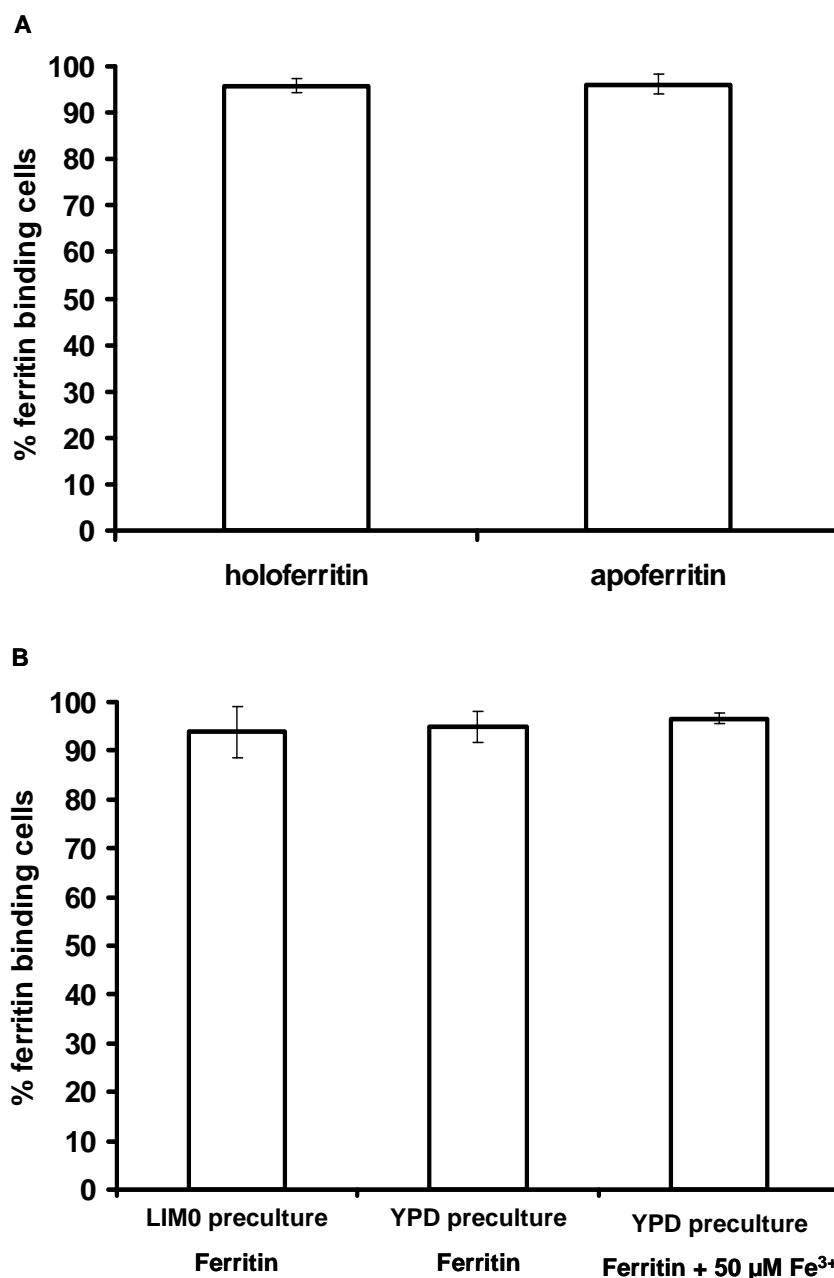


Figure 4.19. Ferritin binding is not iron regulated

A, wild type cells (SC5314) binding holo-ferritin (iron loaded ferritin) or apo-ferritin (empty ferritin molecule). **B**, ferritin binding by cells under iron limitation (preculture in LIM0, then RPMI with ferritin), iron sufficiency (preculture in YPD, then RPMI with ferritin) or iron excess (preculture in YPD, then RPMI with ferritin and iron). All experiments were repeated at least twice and performed in duplicate. Neither iron limitation nor iron excess influenced ferritin binding.

4.3.5. Binding is necessary for iron acquisition from ferritin

In 4.2.3 and 4.3.1 it was demonstrated that *C. albicans* can use iron from ferritin *in vitro* and can bind ferritin. The next question was: is the binding essential for iron acquisition from ferritin? To answer this question, cells from a LIM0 preculture were incubated for 1 h on cover-slips in RPMI medium without ferritin, with holo-ferritin (ferritin with iron) or with apo-ferritin (ferritin without iron). Afterwards the cells were washed four times to remove unbound ferritin, removed from the cover-slips and serial dilutions were spotted on SD agar with or without iron chelator (BPS). Only cells preincubated with holo-ferritin were able to replicate on agar plates with iron chelator.

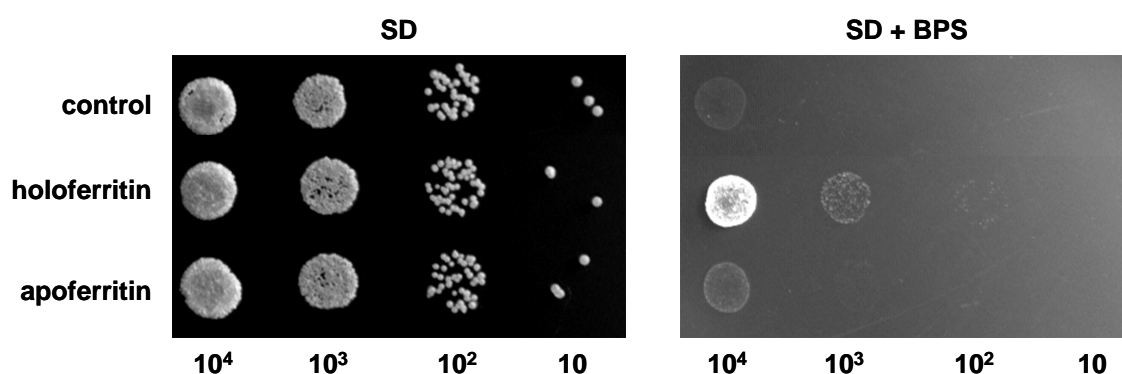


Figure 4.20. Binding of ferritin is necessary for iron acquisition from ferritin

Cells incubated for 4 h on cover-slips in RPMI, RPMI with 100µg holo-ferritin or RPMI with 100µg apo-ferritin. These conditions allowed binding of ferritin as described above. Unbound ferritin was removed by washing. Afterwards, the cells were removed from the cover-slips, diluted and spotted on SD plates with or without 350 µM BPS. All plates were incubated for 3 days at 37°C under 5% CO₂. The assay was performed twice in triplicate. Only *C. albicans* (SC5314) cells, which were incubated under conditions that allowed ferritin binding were able to grow on SD plates with BPS.

4.4. Identification of a ferritin receptor

4.4.1. The search for a ferritin receptor: *in silico* and genetic analysis

The first study to describe a mammalian ferritin receptor has only recently been published (Chen *et al.*, 2005). The murine Tim1 and Tim2 proteins possess T-cell immunoglobulin and mucin domains without clearly defined functions. Chen and co-workers demonstrated that Tim2 is a ferritin receptor that specifically binds to the H-

ferritin subunit and mediates its endocytosis (Chen *et al.*, 2005). Although Tim1 and Tim2 share 65% identity, Tim1 does not function as a ferritin receptor (Chen *et al.*, 2005). Using the protein sequence of Tim2 as a query, a BLASTP search was performed against the *Candida* Genome Database (www.candidagenome.org) to identify putative *C. albicans* ferritin receptor-like proteins. The best hit was a 56 amino acid long fragment from the deduced protein sequence of *RBT1* (**R**epressed **B**y *TUP1*) (Braun *et al.*, 2000). To further investigate if this 56 amino acid sequence within Rbt1 may contain a possible conserved ferritin-binding domain, a ClustalW alignment was performed using the protein sequences from Rbt1 (*C. albicans*), Tim1 (*Mus musculus* and *Rattus norvegicus*) and Tim2 (*M. musculus* and *R. norvegicus*). The Rbt1 fragment showed homology not only to Tim2, but also to Tim1 (Figure 4.21), suggesting that the conserved region shared by the five proteins is unlikely to contain the ferritin-binding domain present in Tim2.

Although *in silico* analysis did not provide strong evidence for a conserved ferritin-binding domain within Rbt1, the corresponding gene *RBT1* is known to be a hyphal-associated gene, which encodes a GPI-anchored cell surface protein essential for *C. albicans* virulence (Bran *et al.*, 2000). Since these characteristics are in agreement with the view that Rbt1 may be a ferritin receptor, a mutant lacking this gene ($\Delta rbt1$) was tested for ferritin binding. However, the majority of $\Delta rbt1$ cells ($92.77\% \pm 1.52\%$) were still able to bind ferritin, excluding the possibility that Rbt1 is directly involved in ferritin binding.

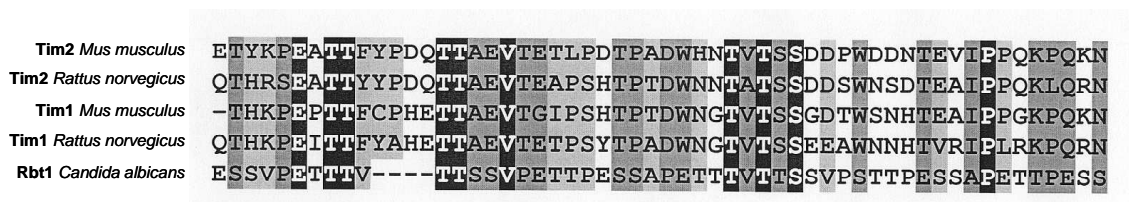


Figure 4.21. *In silico* search for a ferritin receptor

A BLASTP search was performed against the translated *Candida* Genome Database using the protein sequence of Tim2 (mouse ferritin receptor) as a query. A 56 amino acid fragment of Rbt1 showed 33% identity to Tim2 from *M. musculus*. The protein sequences of Rbt1 (*C. albicans*), Tim1 (*M. musculus* and *R. norvegicus*) and Tim2 (*M. musculus* and *R. norvegicus*) were then used for a ClustalW alignment performed with LaserGene5 software. The protein sequence of Tim1 (which does not function as a ferritin receptor) was used as negative control for a possible ferritin-binding domain. Protein sequences of Tim1 and Tim2 were acquired from the NCBI protein database and the protein sequence from Rbt1 was acquired from *Candida* Genome Database.

4.4.2. Transcription profiling of *C. albicans* cells binding ferritin identifies a gene necessary for ferritin binding

Since a mutant lacking Rbt1 – a candidate ferritin receptor, identified by sequence similarities to the only known ferritin receptor – was still able to bind ferritin, a microarray-based approach was chosen to identify putative ferritin receptors encoded by the *C. albicans* genome.

First, the strains CAI4+CIp10 (true hyphae and ferritin binding), *Δhgc1* (no true hyphae and ferritin binding) and *Δras1* (no hyphae and no ferritin binding) were incubated for 1.5 h under hyphal induction conditions in the presence of ferritin. Afterwards, the RNA was isolated, labelled and hybridised on a *C. albicans* microarray. It was assumed that a gene encoding a ferritin receptor should be up-regulated in wild type hyphal cells and *Δhgc1* cells under hyphal inducing conditions, but should not be up-regulated in *Δras1* cells under hyphal inducing conditions. Using this approach, a total of 22 genes were selected as encoding possible ferritin receptor candidates (Figure 4.22 and Table 4.2). Three of these genes were known to encode hyphal-specific proteins, which are cell surface localised as expected for a receptor protein. Consequently, these three genes were further investigated.

It should be mentioned that the *URA3* gene was reconstituted in all three strains using the plasmid CIp10, maintaining an isogenic background (section 3.17).

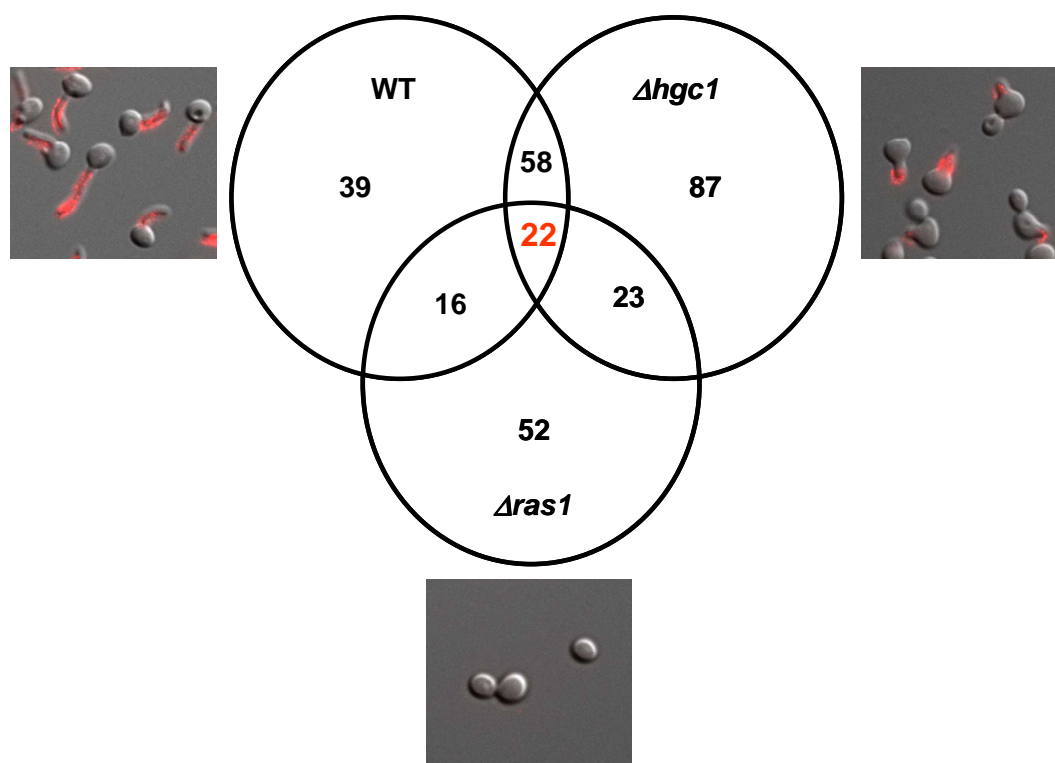


Figure 4.22. Transcription profiling identifies genes associated with ferritin binding.

To identify genes necessary for ferritin binding, *C. albicans* wild type, $\Delta hgc1$ and $\Delta ras1$ mutant cells were incubated under conditions where ferritin bound to wild type and $\Delta hgc1$, but not $\Delta ras1$ cells. RNA of each population of cells was isolated and used for microarray analysis. The micrographs show representative anti-ferritin labelled cells at the time point of RNA isolation. The Venn diagram indicates the number of genes up-regulated in wild type and $\Delta hgc1$ and either unchanged or down-regulated in $\Delta ras1$, as compared to a common control. Twenty two genes were up-regulated in wild type and $\Delta hgc1$, but not $\Delta ras1$ cells as expected for a ferritin receptor. Microarray experiments were performed in four biological replicates (two of them using dye swap). Note that the schematic presentation of the Venn diagram combines up-regulated (wild type and $\Delta hgc1$) and unaltered or down-regulated ($\Delta ras1$) genes to clarify the selection strategy.

Table 4.2. Genes up-regulated in wild type and *Δhgc1* cells, but unaltered or down-regulated in the *Δras1* mutant

Gene name	Fold up-regulated	Description
ECE1	22.0	cell elongation protein
UME6	6.6	transcription factor
PUT2	5.3	1-pyrroline-5-carboxylate dehydrogenase (by homology)
ALS3	5.0	agglutinin like protein
orf19.4805	5.0	unknown function
FAS2	4.4	fatty-acyl-CoA synthase (internal fragment)
ADO1	3.8	adenosine kinase (by homology)
orf19.2210	3.4	unknown function
FAS1	3.3	fatty-acyl-CoA synthase
ABC1	3.2	acyl-CoA binding (by homology)
ACC1	3.2	acetyl-CoA carboxylase (by homology)
ERG25	3.2	C-4 methylsterol oxidase (by homology)
orf19.4468	3.0	succinate dehydrogenase (by homology)
orf19.5147	2.9	unknown function
UAP1	2.8	UDP-N-acetylglucosamine pyrophosphorylase
orf19.801	2.8	unknown function
FAS2	2.7	fatty-acyl-CoA synthase (3-prime end)
HYR1	2.6	hyphally regulated protein
orf19.5126	2.6	unknown function
RPS9B	2.6	ribosomal protein (by homology)
orf19.2650.1	2.6	mitochondrial ribosomal protein (by homology)
orf19.1186	2.5	unknown function

Genes encoding known hyphal surface proteins are in red.

4.4.3. Deletion of *ALS3* precludes ferritin binding

The three genes identified in the analysis described in 4.4.2 which encode cell surface localized and hyphal-associated proteins were *ECE1*, *HYR1* and *ALS3* (marked red in Table 4.2). *ECE1* (**E**xtent of **C**ell **E**longation) is a hyphal-specific gene with as yet unknown function. Expression of *ECE1* increases during elongation of hyphal cells. The gene encodes a predicted cell membrane protein and the corresponding knockout mutant displays no obvious altered phenotype (Birse *et al.*, 1993). *HYR1* (**HY**phally **R**egulated) encodes a GPI-anchored protein, predicted to be cell wall localized (Bailey *et al.*, 1996). A specific function for this protein has yet to be identified. Finally, *ALS3* (**A**gglutinin-**L**ike **S**equences) encodes a hyphal-specific GPI-anchored cell wall protein, which belongs to the Als family of adhesins (Hoyer, 2001) and which plays a crucial role in epithelial and endothelial adhesion (Zhao *et al.*, 2004) and invasion (Phan *et al.*, 2007). The corresponding knockout mutants were tested for ferritin binding. Both the *Δece1* and the *Δhyr1* mutants efficiently bound ferritin at similar rates compared to the wild

type. In contrast, the $\Delta als3$ mutant completely lost its ability to bind ferritin. An $\Delta als3 + ALS3$ revertant strain reconstituted the wild type ferritin binding phenotype (Figure 4.23). This result suggested that Als3 plays a crucial role in ferritin binding and may in fact be the sought ferritin receptor.

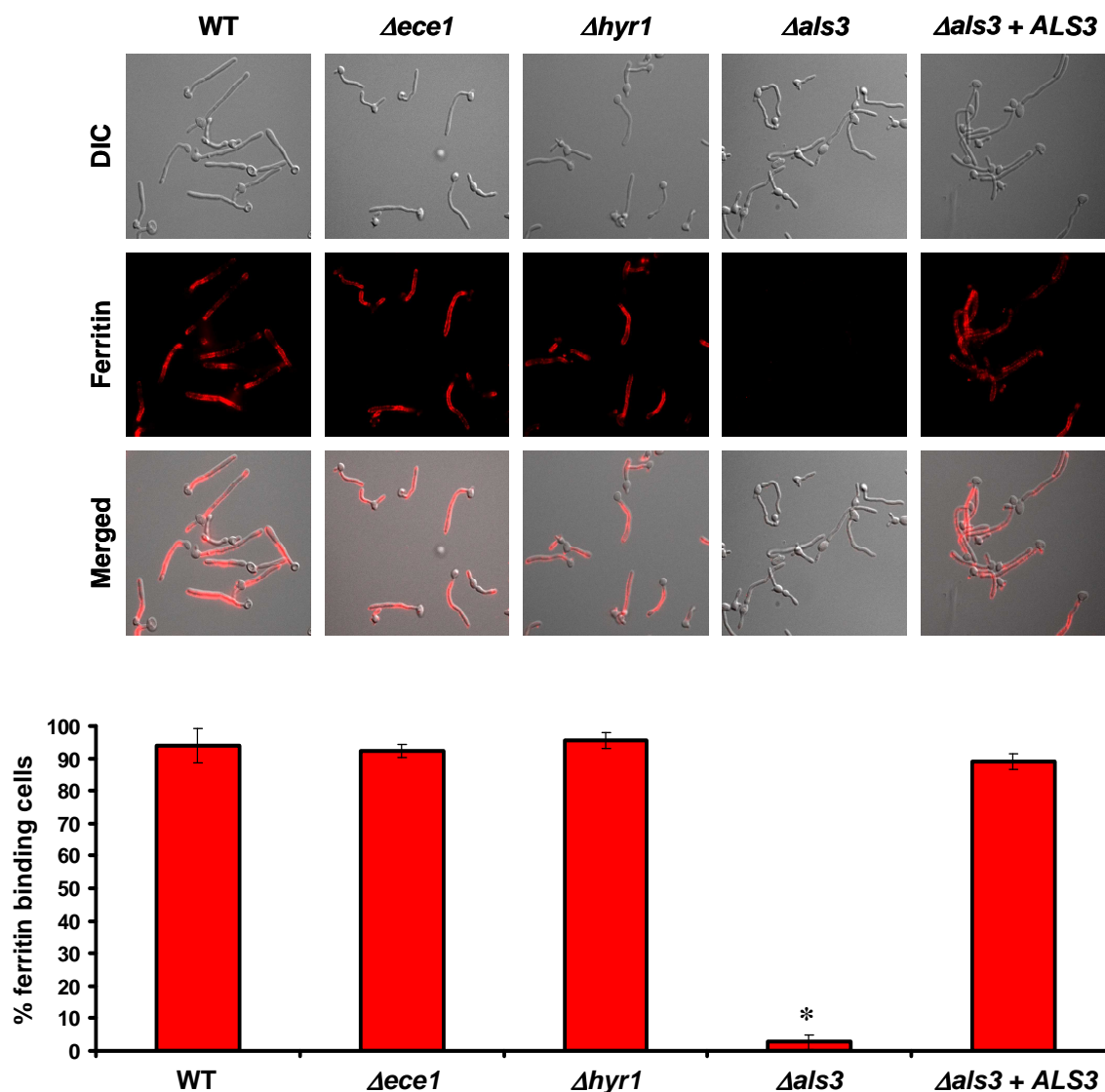


Figure 4.23. Als3 is essential for ferritin binding

Mutants lacking either *ALS3*, *HYR1* or *ECE1* - the three selected genes predicted to encode ferritin receptors - were tested for ferritin binding. Bar indicates 10 μ m. The ferritin binding was quantified by counting >100 randomly selected cells using fluorescence microscopy. The $\Delta als3$ mutant lost its ability to bind ferritin, while an $\Delta als3 + ALS3$ revertant strain bound ferritin similar to the wild type. * indicates significant difference compared to wild type ($p < 0.0001$). WT, *C. albicans* wild type (SC5314).

4.4.4. Upstream regulators of *ALS3* are required for ferritin binding

If Als3 is indeed a ferritin receptor, it would be expected that mutants lacking transcriptional regulators of this gene would also have an altered capacity to bind ferritin. Therefore, two mutants, which lack transcriptional regulators involved in the regulation of *ALS3*, were tested. *BCR1* codes for a transcription factor which regulates the expression of certain hyphal-specific genes, including *ALS3* (Nobile *et al.*, 2006). Furthermore, expression of *BCR1* itself depends upon Tec1 (Nobile and Mitchell, 2005). Figure 4.24 shows that the presence of both transcriptional factors, Tec1 and Bcr1, is necessary for *C. albicans* cells to bind ferritin. These data reinforce the view that Als3 is likely a ferritin receptor.

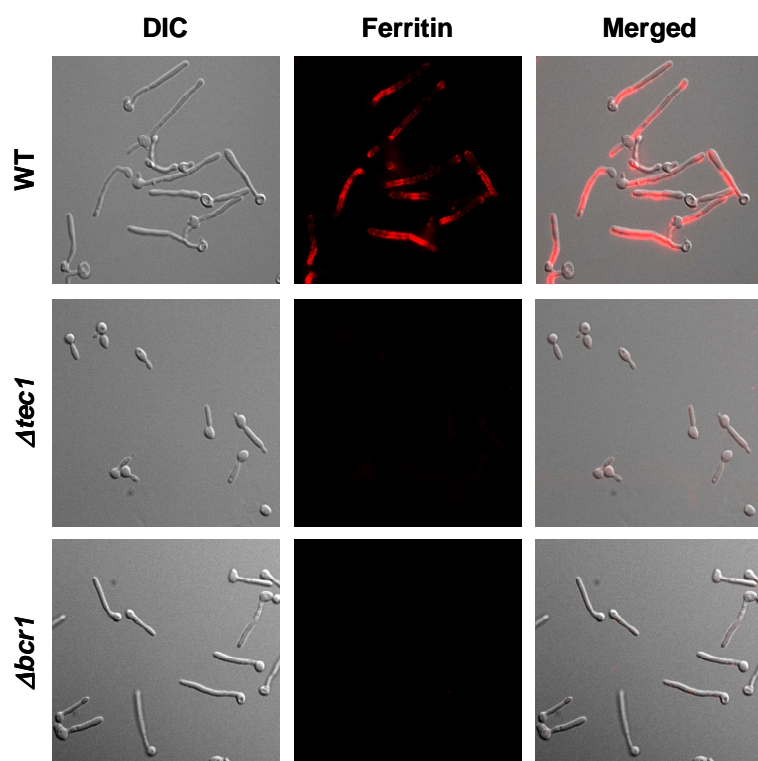


Figure 4.24. Upstream regulation of Als3 is necessary for ferritin binding

Ferritin binding assay with mutants lacking key regulators of *ALS3* expression (*Δtec1* and *Δbcr1*). Both mutants had reduced abilities to bind ferritin. Bar indicates 10 μm. WT, *C. albicans* wild type (SC5314).

4.4.5. Als3 is a ferritin receptor

Since the $\Delta als3$ mutant lost its ability to bind ferritin, it is possible that Als3 functions as a ferritin receptor. To verify whether Als3 itself can facilitate ferritin binding without additional *C. albicans* surface factors, a strain of *S. cerevisiae* overexpressing *ALS3* was tested for ferritin binding. Because, *ALS3* is a member of a large gene family encoding similar proteins, two further *S. cerevisiae* strains (overexpressing *ALS1* or *ALS5*) were also tested. If one of the corresponding proteins is a ferritin receptor, it would be expected that the *S. cerevisiae* strain overexpressing this protein on the surface would be able to bind ferritin. Only the strain overexpressing Als3 strongly bound ferritin (Figure 4.25). Strains expressing Als1 or Als5 did not show any detectable binding of ferritin demonstrating that two other members of the *ALS*-family are not involved in this process (Figure 4.25).

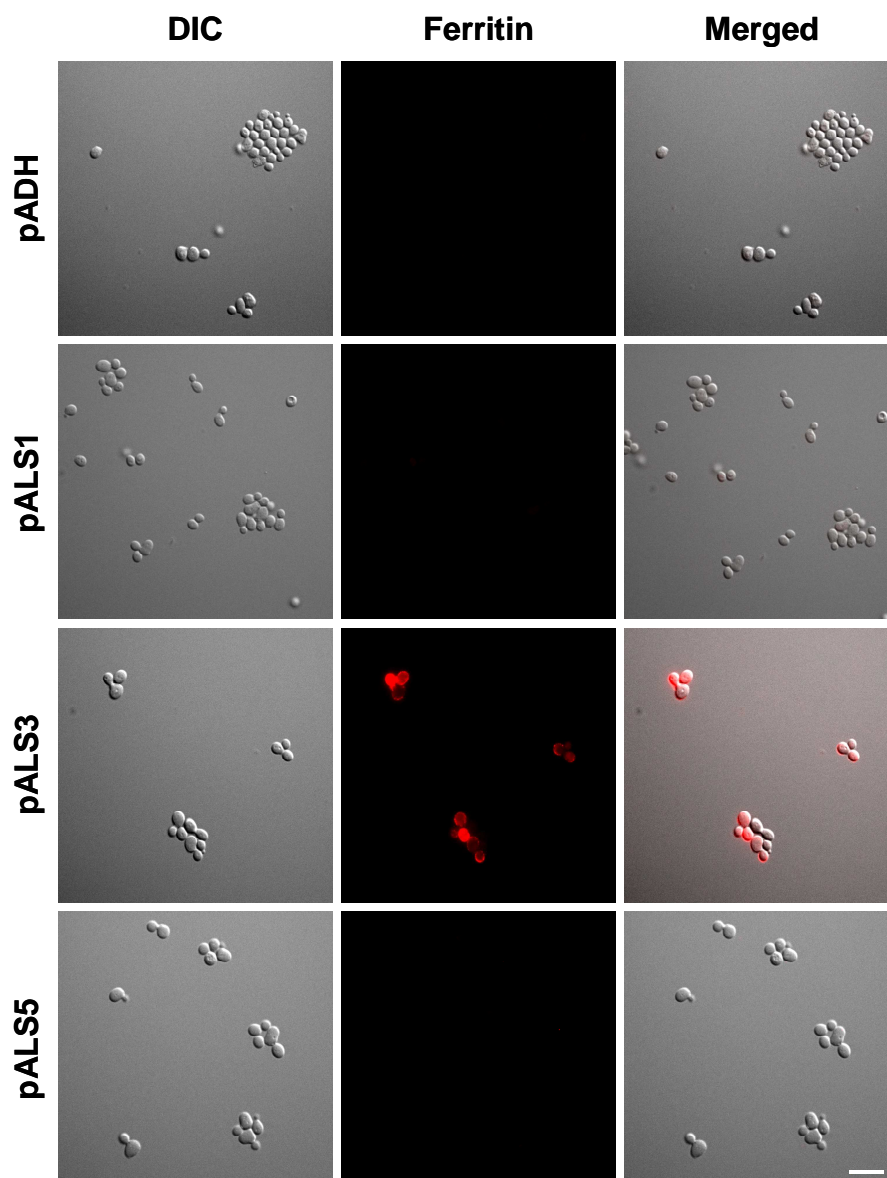


Figure 4.25. Als3 is a ferritin receptor

S. cerevisiae cells overexpressing *ALS1*, *ALS3*, *ALS5* (driven by the ADH promoter) or carrying an empty plasmid (pADH) were incubated for 15 min in the presence of 25 $\mu\text{g/ml}$ apoferritin coupled to a fluorescent dye. Cells were washed to remove non-bound ferritin and analysed with fluorescence microscopy in duplicate repeated three times. Only *S. cerevisiae* cells expressing Als3 bound ferritin. Bar indicates 10 μm .

4.5. Analysis of a mutant lacking the ferritin receptor

4.5.1. The $\Delta als3$ mutant has no general iron uptake defect

To investigate if the lack of Als3 could affect high-affinity iron uptake and consequently influence further experiments, an iron uptake assay was used. The addition of 120 μ M BPS to YPD agar allowed growth of *C. albicans* wild type cells (positive control), but under these conditions the iron permease mutant ($\Delta ftr1$) – defective in high-affinity iron uptake – was not able to grow (negative control). As shown in Figure 4.26, $\Delta als3$ and its revertant strain were still able to grow under iron limitation caused by the addition of 120 μ M BPS, demonstrating that the $\Delta als3$ mutant had no defect in high-affinity iron uptake.

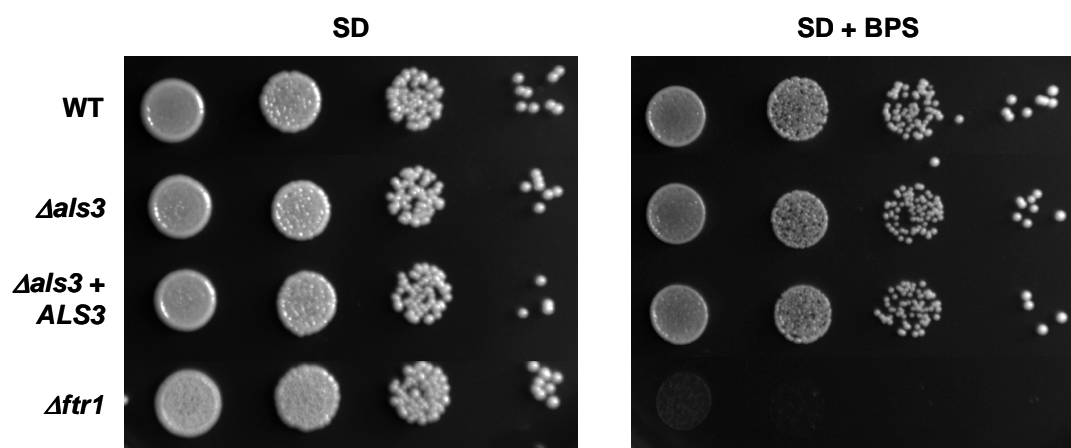


Figure 4.26. The $\Delta als3$ mutant has no defect in uptake of free iron

The addition of 120 μ M BPS abolished growth of the $\Delta ftr1$ mutant, but not growth of the wild type, the $\Delta als3$ mutant or the $\Delta als3 + ALS3$ revertant strain. Under these conditions, only cells with a functional high-affinity iron uptake system were able to grow. The plates were incubated for 2 days at 37°C.

4.5.2. *C. albicans* cells lacking Als3 lost their ability to grow with ferritin as the sole source of iron

In this study it was shown that *C. albicans* grew on ferritin plates, bound ferritin and that this binding was necessary for iron utilization from ferritin. The next question was: is the binding via Als3 essential for iron acquisition from ferritin? To answer this question, a mutant lacking *ALS3* was tested for growth on ferritin plates.

In fact, the $\Delta als3$ mutant displayed impaired growth on agar plates (pH 7.4) with ferritin as the sole source of iron (Figure 4.27). Reconstitution of one copy of the gene, improved its growth, but not to wild type levels. These results demonstrated that binding of ferritin by Als3 is necessary to promote *C. albicans* growth on ferritin plates.

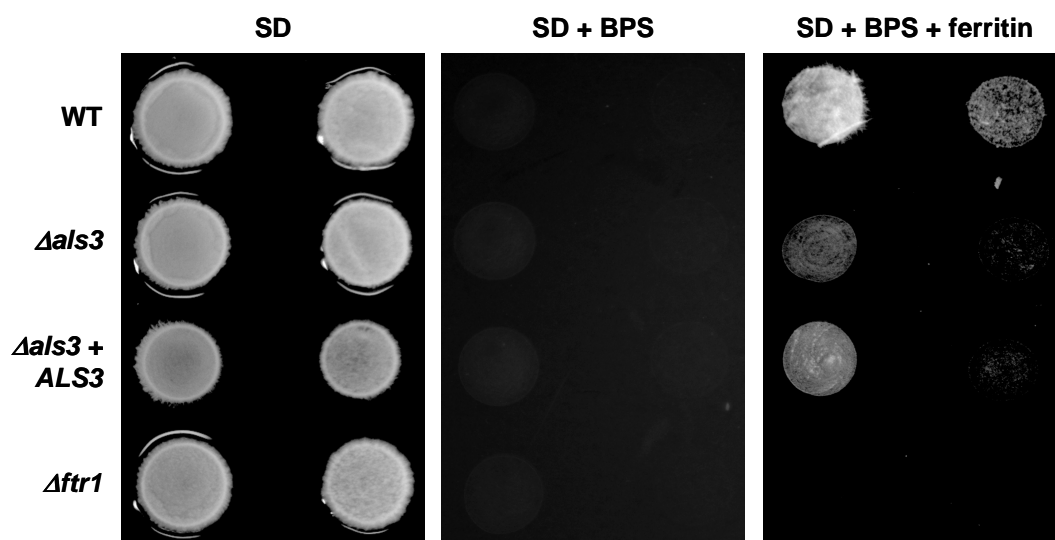


Figure 4.27. Binding via Als3 is necessary for iron acquisition from ferritin

C. albicans wild type (CAF2-1), $\Delta als3$, $\Delta als3 + ALS3$ and $\Delta ftr1$ strains were grown on media containing ferritin as the sole source of iron. SD agar was buffered using 100 mM HEPES (pH 7.4). BPS, iron chelator; ferritin, 2 μ g/ml holo-ferritin. Cells were spotted at two concentrations (left to right, 10^5 and 10^4 cells, respectively) for each strain. All plates were incubated for 3 days at 37°C under 5% CO₂. The assay was performed three times. The mutant lacking Als3 had strongly reduced ability to grow with ferritin as the sole source of iron.

4.5.3. Invading *C. albicans* hyphae bind ferritin from epithelial cells during infection

Next, it was investigated whether ferritin binding via Als3 occurs when *C. albicans* interacts with host cells. Oral epithelial cells were loaded with iron and then incubated with wild type *C. albicans*, the $\Delta als3$ mutant, or the $\Delta als3 + ALS3$ revertant strain for 6 h.

To localize ferritin molecules during oral epithelial cell infection with *C. albicans*, an immunofluorescence approach was used, which allowed discrimination between hyphae located on the epithelial cell surface and hyphae that had invaded into the epithelial cells (Figure 4.28 columns 1, 2 and 4). In addition, ferritin was localized with an anti-ferritin antibody (Figure 4.28 column 3). As shown in Figure 4.28, hyphae of wild type

and $\Delta als3+ALS3$ revertant strains invaded the epithelial cells and were surrounded by ferritin (white arrows in Figure 4.28). In contrast, the few hyphae of the $\Delta als3$ mutant that had invaded the epithelial cells displayed no accumulation of ferritin (Figure 4.28G and 4.28K). These results indicated that invading *C. albicans* hyphae accumulated ferritin on the cell surface and that Als3 is necessary for this accumulation.

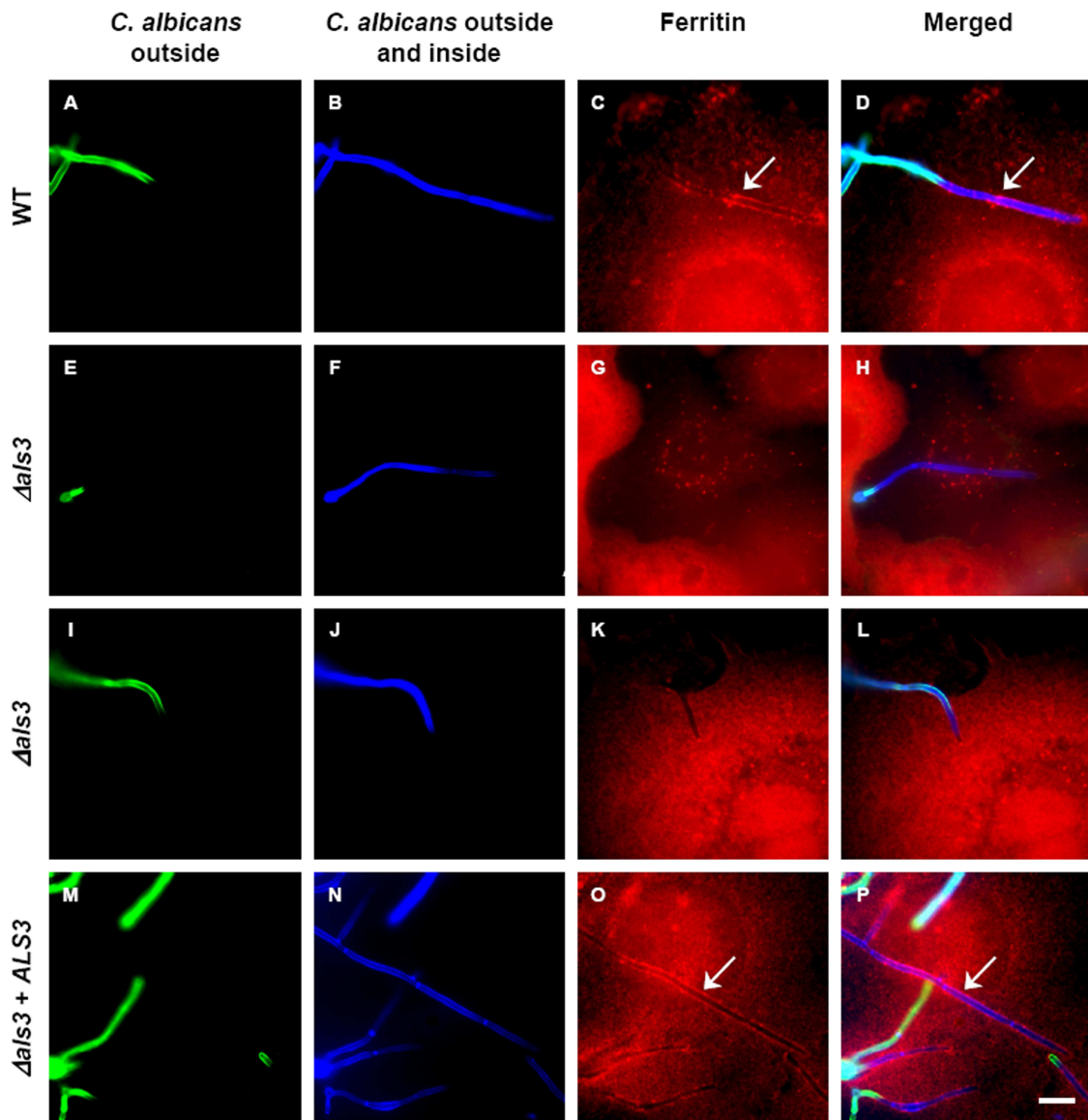


Figure 4.28. *C. albicans* hyphae invading oral epithelial cells bind ferritin

C. albicans wild type (SC5314), $\Delta als3$ mutant and $\Delta als3+ALS3$ revertant cells were co-incubated with ferritin-enriched oral epithelial cells and differentially stained. **A, E, I and M** - staining of extracellular (non-invading) *C. albicans* with concanavalin A conjugated with fluorescein before cell permeabilization. **B, F, J and N** - calcofluor white staining of whole *C. albicans* cells following epithelial cell permeabilization. **C, G, K and O** - fluorescent dye (DY649) coupled antibody staining of ferritin. White arrows indicate hyphae surrounded by epithelial ferritin. Note the accumulation of ferritin around hyphae from cells containing Als3. **D, H, L and P** - merged images. Bar in **P** indicates 10 μm .

4.5.4. *C. albicans* mutants lacking genes essential for iron utilization from ferritin are unable to damage epithelial cells

If binding to ferritin and utilizing host iron are important for *C. albicans* to cause an oral infection, it would be expected that mutants lacking *ALS3* or *FTR1* would have a reduced potential to cause tissue damage as compared to wild type cells. To verify this hypothesis, the extent of epithelial cell damage caused by wild type, $\Delta als3$ and $\Delta ftr1$ mutant strains of *C. albicans* were tested. As showed in Figure 4.29, the $\Delta als3$ and $\Delta ftr1$ mutants lost their capacity to damage oral epithelial cells.

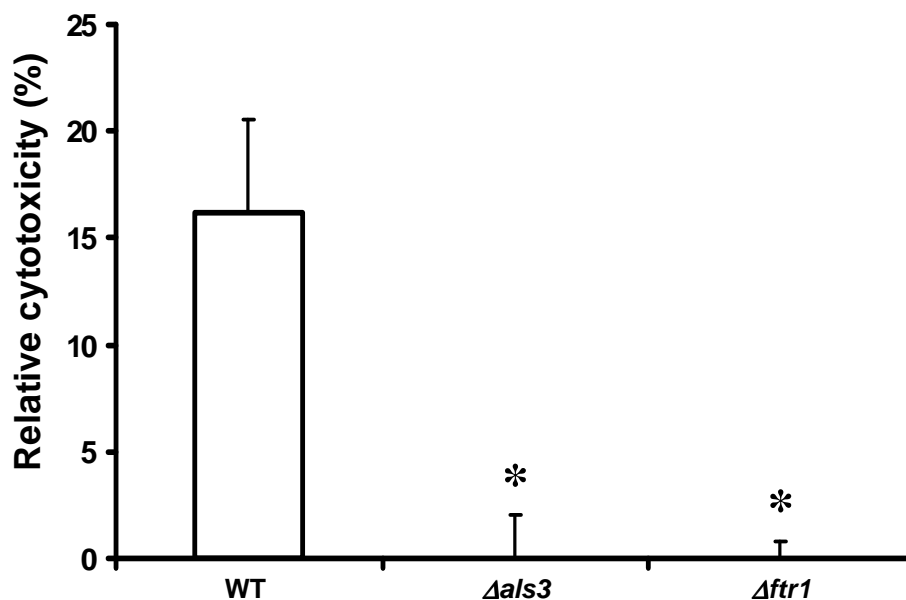


Figure 4.29. Iron uptake from ferritin plays a role in oral epithelial cell damage

C. albicans wild type (CAF2-1), $\Delta als3$ mutant and $\Delta ftr1$ mutant cells were co-incubated with oral epithelial cells. The monolayers were incubated for 8 h in serum-free RPMI with 10^6 *C. albicans* cells and cell damage was quantified by monitoring the release of epithelial LDH into the medium. The experiment was performed five times in triplicate. Both $\Delta als3$ and $\Delta ftr1$ mutant cells were unable to cause cytotoxicity. * indicates significant difference compared to the wild type ($p < 0.0001$).

4.6. Cellular dissection of the interactions between *C. albicans* and oral epithelial cells

4.6.1. Fluorescence microscopy analysis of *C. albicans* infecting epithelial cells reveals a novel phenomenon termed the “glove effect”

During the immunofluorescence approaches used to analyse if *C. albicans* may be able to bind ferritin during invasion of epithelial cells (section 4.5.3), it was noted that wild type *C. albicans* hyphae, invading epithelial cells, may be able to stretch the epithelial cell membranes without disrupting these cells. As shown in Figure 4.30, the hyphal cell, surrounded by ferritin, appeared to elongate from the edge of the host cell for 28.54 μM without being exposed to the extracellular space.

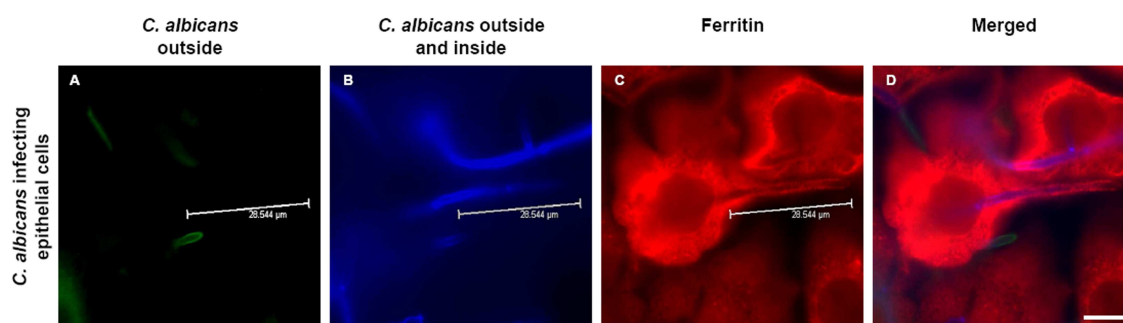


Figure 4.30. Fluorescence microscopy analysis of *C. albicans* infecting epithelial cells

C. albicans wild type (SC5314) cells were co-incubated with ferritin-enriched oral epithelial cells for 6 h and differentially stained. **A**, staining of extracellular (non-invading) *C. albicans* with concanavalin A conjugated with fluorescein before cell permeabilization. **B**, calcofluor white staining of whole *C. albicans* cells following epithelial cell permeabilization. **C**, anti-ferritin immuno-staining (Alexa Fluor® 680). **D**, merged images. Bar in **D** indicates 10 μm .

To analyse if the *C. albicans* hyphal cells with surrounded ferritin (Figure 4.30D) were indeed stretching the epithelial cell membranes, a fluorescent staining of plasma membranes was performed. As shown in Figure 4.31F and J, *C. albicans* can stretch the epithelial cell membranes (white arrows). Because the stretched epithelial cell membranes resemble a finger wearing a glove, this phenomenon was termed the “glove effect”.

Additionally, *C. albicans* hyphal cells were able to escape from one epithelial cell (Figure 4.31J), reach the extracellular space (the hyphal part in green indicate by the red arrow) and subsequently invade the neighbouring host cell.

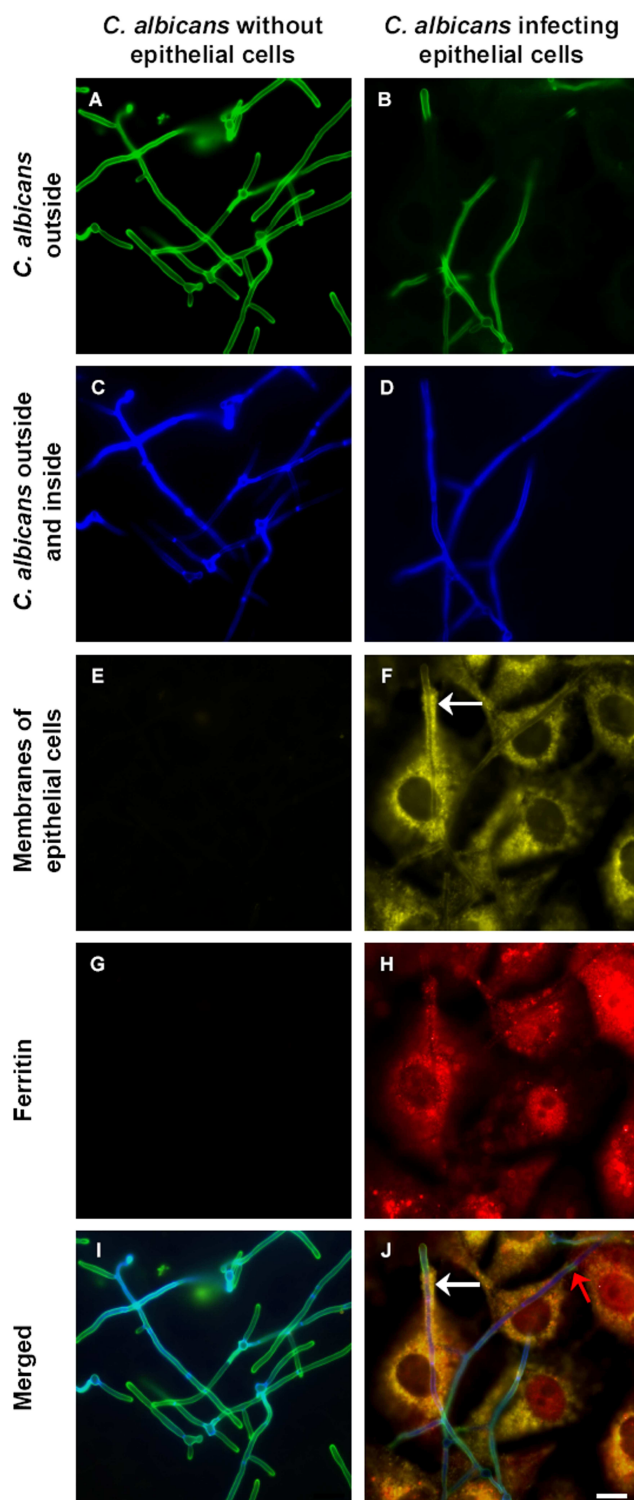


Figure 4.31. *C. albicans* can stretch epithelial cell membranes during infection

C. albicans wild type (SC5314) cells were incubated without (control) or with oral epithelial cells for 6 h and differentially stained. **A** and **B** - staining of extracellular (non-invading) *C. albicans* with concanavalin A conjugated with fluorescein before cell permeabilization. **C** and **D** - calcofluor white staining of whole *C. albicans* cells following epithelial cell permeabilization. **E** and **F** – epithelial cell membranes stained with CM-Dil. **G** and **H** - anti-ferritin immuno-staining (Alexa Fluor® 680). **I** and **J** - merged images. White arrows indicate the *C. albicans*-induced stretched epithelial plasma membranes (“glove effect”). Red arrow points the part of a hyphal cell (in green) between two trespassed epithelial cells. Bar in **J** indicates 10 μ m.

4.6.2. The “glove effect” depends on the iron status of the epithelial cells

The “glove effect” phenomenon was first visualised in ferritin-enriched epithelial cells co-incubated with *C. albicans*. To further analyse this phenomenon, ferritin-enriched, ferritin-depleted or non-treated (see section 4.2.1) epithelial cells were co-incubated for 6 h with wild type *C. albicans* (SC5314), differentially stained as shown in Figure 4.31, and analysed under the fluorescence microscope. The “glove effect” was frequently observed following co-incubation of *C. albicans* with ferritin-enriched epithelial cells (Figure 4.32A, C and E) but was a rare event in non-treated cells. In contrast, no “glove effect” was observed following co-incubation of *C. albicans* with ferritin-depleted epithelial cells (Figure 4.32B, D and F). These results suggested that the “glove effect” phenomenon is influenced by the iron/ferritin status of the host cells.

Additionally some interactions between the fungus and the epithelial cells were observed. The white arrows (Figure 4.32) show *C. albicans* hyphae trespassing the epithelial cells nuclei. The red arrow in Figure 4.32D indicates a hyphal cell in the intercellular space between two epithelial cells. Finally, the red arrow in Figure 4.32F indicates epithelial pseudopodia enveloping a *C. albicans* hyphal cell.

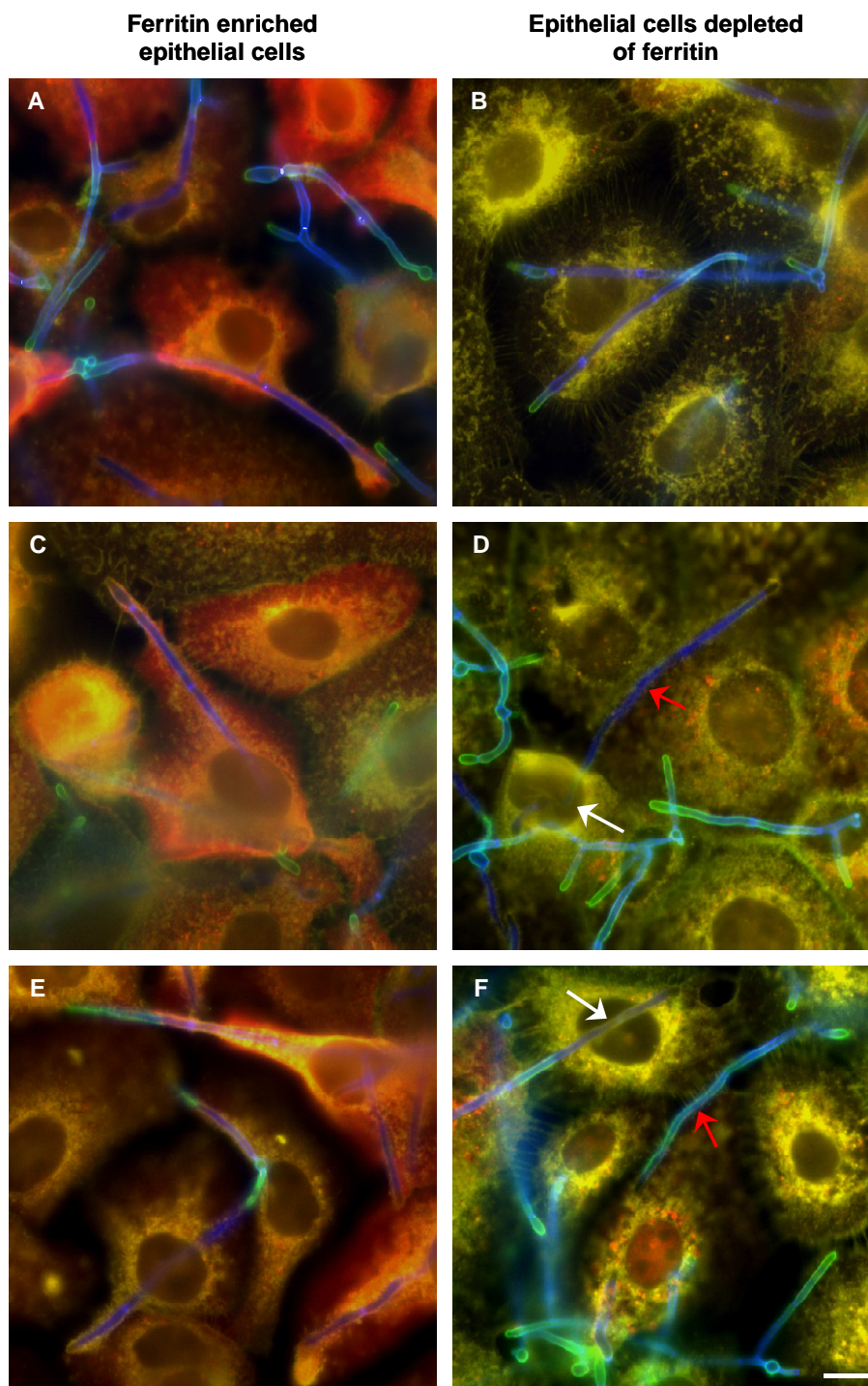


Figure 4.32. The iron status of host epithelial cells influences their interactions with *C. albicans*
C. albicans wild type (SC5314) cells were co-incubated with ferritin-enriched or ferritin-depleted oral epithelial cells for 6 h and differentially stained. **A, C and E** - *C. albicans* co-incubated with ferritin-enriched oral epithelial cells. **B, D and F** - *C. albicans* co-incubated with ferritin-depleted oral epithelial cells. The immunofluorescence micrographs show merged images of the differential stain described in Figure 4.31. Green/cyan, extracellular (non-invading) *C. albicans*. Blue, *C. albicans* inside (invading) oral epithelial cells. Yellow, oral epithelial cell membranes. Red, epithelial cell ferritin. White arrows point to hyphae perforating the epithelial cells nuclei. Red arrow in **D** points a hyphal cell in the intercellular space between two epithelial cells. Red arrow in **F** points epithelial cell pseudopodia enveloping a hyphal cell. Note that the “glove effect” phenomenon is visualised only following co-incubation of *C. albicans* with ferritin-enriched epithelial cells (**A, C and E**). Bar in **F** indicates 10 μm .

5. Discussion

5.1. *C. albicans* needs iron for growth and has intracellular iron storage

Iron, as an essential cofactor for several proteins, is required for numerous biochemical processes including cellular respiration and metabolism, oxygen transport, drug metabolism and DNA synthesis (Welch *et al.*, 2001). In the microbial world, there is constant competition for iron, both between microbes within microbial communities and between microbes and their hosts during host-pathogen interactions. Since this thesis aimed to identify the natural iron sources and the iron acquisition systems used by *C. albicans* during oral infection, the first basic experiments were designed to investigate the influence of iron limitation on *C. albicans* growth. As already shown in a series of previous studies, the experiments mentioned above indicated that iron is required for the growth and proliferation of both hyphal and yeast forms of *C. albicans* (Eck *et al.*, 1999; Weissman *et al.*, 2002; Han, 2005; Lee and Han, 2006).

However, although iron limitation strongly inhibited proliferation of *C. albicans*, yeast growth and germ tube formation were not completely blocked. *C. albicans* cell division was only completely blocked when both intracellular and extracellular iron was chelated with bipyridine. These data suggested that *C. albicans* may possess an intracellular iron storage capacity, which allows low levels of cell division or germ tube formation under iron limitation. Consistent with this, it was previously reported that *C. albicans* cell division was only totally blocked after five passages (of 5 days incubation each) in iron limited (LIM) medium supplemented with BPS (extracellular iron chelator) (Eck *et al.*, 1999). Eck and co-workers also proposed intracellular iron storage as the mechanism by which *C. albicans* can continue to divide in the absence of extracellular iron. Cellular mechanisms by which iron is stored intracellularly have been demonstrated in other fungi. For example, polyphosphates have been implicated in vacuolar iron storage within *S. cerevisiae* cells; and among the zygomycetes, ferritin-like proteins (mycoferritins) function as iron storage molecules (Howard, 1999). *A. fumigatus* uses the siderophores ferricrocin and hydroxyferricrocin for hyphal and conidial iron storage, respectively (Schrettl *et al.*, 2007). However, the molecular mechanisms by which *C. albicans* is able to store iron intracellularly have yet to be investigated.

5.2. Ferritin, a novel iron source used by *C. albicans* during oral infections

If *C. albicans* requires iron for growth and extracellular niches within a host are virtually iron-free, how can the fungus grow and proliferate during oral infections? As discussed above, fungal intracellular storages would allow only slow proliferation and these stores also need to be refilled by external iron sources.

In this study it was observed that oral epithelial cells enriched in intracellular ferritin were more susceptible to tissue damage by *C. albicans* and that epithelial cells depleted of ferritin were significantly protected from damage, suggesting that the ferritin status of oral cells has a direct impact on the fungus to damage these cells. Although this is the first study which demonstrates a correlation between the ferritin content of host cells and host cell damage, other studies have reported that the general iron content of a host or host cells has an influence on *C. albicans* virulence and cell damage. For example, iron overload in mice increased *C. albicans* virulence in a model of systemic candidosis (Abe *et al.*, 1985) and endothelial cells incubated with an iron chelator before *C. albicans* infection were shown to be protected from injury by *C. albicans* (Fratti, *et al.*, 1998). Moreover, the anti-*Candida* activity of ciclopirox olamine, a potent antifungal agent, is proposed to be caused by iron chelation (Niewerth *et al.*, 2003; Lee *et al.*, 2005; Sigle *et al.*, 2005). In general, it can be concluded that access to iron has a direct influence on the pathogenicity of *C. albicans*. Moreover, the direct correlation between cellular ferritin content and damage, in combination with several other experiments described in this study, suggests that *C. albicans* is able to utilise ferritin during infection of epithelial cells. The ability to do so and the mechanisms by which this usage is facilitated are unique pathogenicity mechanisms. Ferritin is an extremely robust and stable protein which, so far, has not been reported to be degraded by microbial activity. In fact, *N. meningitidis* is the only microorganism that has so far been shown experimentally to exploit holoferitin as an iron source during interaction with host cells (Larson *et al.*, 2004). However, this bacterium is not able to directly utilise iron from ferritin, but rather induces degradation of cytosolic ferritin by manipulating the host cellular machinery and utilises the resultant free cytosolic iron: no published studies have demonstrated direct use of iron from host ferritin.

Nevertheless, a number of studies have suggested that certain microbial pathogens can use ferritin as an iron source during *in vitro* growth. For example, *Yersinia pestis* can grow on agar containing hemin, myoglobin, hemoglobin or ferritin (Sikkema and

Brubaker, 1989). A siderophore produced by *M. tuberculosis* (exochelin) can sequester iron from transferrin, lactoferrin and to a lesser extent from ferritin (Gobin and Horwitz, 1996). *Listeria monocytogenes* and *Burkholderia cenocepacia* can grow in liquid medium with ferritin as the sole source of iron (Jin *et al.*, 2006; Whitby *et al.*, 2006). However, the molecular mechanisms of iron acquisition from ferritin by these organisms remain unknown and it is not clear whether ferritin from host cells can be used by any of these species. Furthermore, although ferritin seems to be almost indestructible under physiological conditions, iron may be released from a pool of ferritin molecules when used at high concentrations under non-physiological conditions *in vitro*. In all reports cited above, a commercial ferritin solution (horse spleen ferritin) was used for growth experiments. In this study, it was shown that a commercial ferritin solution contained free iron contamination (see 4.2.2). Therefore, spontaneous iron release from ferritin (by acidification) or free iron contamination will have influenced the results, unless the pH of the media was adjusted to 7.4 (physiological pH) and ferritin solutions were purified before use. This is supported by the fact that the baker's yeast *S. cerevisiae* was able to grow on ferritin plates when the initial pH was adjusted to 5.0. In contrast, *S. cerevisiae* was not able to grow *in vitro* with purified ferritin as the sole source of iron at physiological pH, indicating that the ability to utilise ferritin is also not a generic fungal trait.

5.3. The molecular mechanisms of ferritin exploitation by *C. albicans*

In contrast to *S. cerevisiae*, *C. albicans* was able to use ferritin as the sole source of iron *in vitro* when the growth medium was buffered to physiological pH. Which mechanisms and activities are involved in iron acquisition from ferritin?

Since it is known that *C. albicans* can secrete aspartic proteases (Saps) with very broad substrate specificity (Naglik *et al.*, 2004), it was hypothesised that ferritin could be degraded by extracellular proteolytic activity. However, it appears that extracellular degradation due to fungal proteases is not necessary for growth with ferritin, since mutants lacking the protease genes *SAP1-3* or *SAP4-6* were still able to grow on such media. Indeed, an earlier study by Ruchel demonstrated that ferritin was the only tested protein which was resistant to proteolysis by Sap2, one of the major secreted proteases of *C. albicans* with an extremely broad substrate specificity (Ruchel, 1981), supporting

the view that proteases are not involved in the ability of *C. albicans* to utilize iron from ferritin.

The fact that even *S. cerevisiae* was able to grow with ferritin when the pH of the medium was low (pH 5.0), suggested that pH plays an important role in the release of iron from ferritin. Moreover, *C. albicans* was able to actively lower the pH of neutral/alkaline media to below pH 4.0 (Figure 4.10). In fact, *C. albicans* was only able to use ferritin as an iron source under conditions which permitted acid production – glucose, but not casamino acids as a carbon source and low buffer capacity. These results suggest that iron acquisition from ferritin by *C. albicans* is pH-mediated. Similarly, it has been observed that, under iron starvation, the bacterial pathogen *Staphylococcus aureus* decreases the local pH, resulting in the release of iron from transferrin (Friedman *et al.*, 2006).

Another possible explanation for the results discussed above is that *C. albicans* can produce and secrete reductants, which are able to sequester iron from ferritin. Additionally, this process should be favoured by acidification of the surrounding media. In agreement with this model, reductants/chelators such as thioglycolic acid, ascorbate, aceto- and benzohydroxamic acids are capable of releasing iron from the ferritin core (Joo *et al.*, 1990; Laulhere *et al.*, 1996; Galvez *et al.*, 2005). Underscoring the importance of pH in the release of iron from ferritin, this process was increased at pH 5.2 in comparison to pH 7.4 (Galvez *et al.*, 2005).

An endocytic mechanism for ferritin acquisition, as recently described for haemoglobin utilisation is a further possibility (Weissman *et al.*, 2008). In this scenario, ferritin would be endocytosed and vesicle-transported to an internal compartment, such as the lysosome, where iron is released due to low pH and lysosomal enzyme activities. If this was the case, one would expect the presence of ferritin particles transiting across the cell wall. However, using electron microscopy, ferritin particles were only detected on the external layer of the hyphal cell wall, suggesting that the ferritin molecule itself is not transported into the cell (Fig. 4.17).

Although there is no direct experimental evidence that local acidification occurs *in vivo*, transcriptional profiling of *C. albicans* during experimental intraperitoneal infection suggests that the local environment does in fact change from neutral to acidic pH during invasion and tissue damage, indicated by the up-regulation of the acid induced gene *PHR2* (Thewes *et al.*, 2007).

In addition to the ability to acidify the environment, it was shown that *C. albicans* requires the reductive high-affinity iron uptake pathway to exploit iron from ferritin. Mutants lacking either the high-affinity permease Ftr1 or the copper permease Ccc2 (which is essential for the reductive pathway) (Ramanan and Wang, 2000; Weissman *et al.*, 2002) did not grow on ferritin plates, even when the initial pH was low. The high affinity pathway, however, is not essential for iron acquisition from other sources; for example, it was shown that the $\Delta ftr1$ mutant was still able to grow with haemoglobin as the sole source of iron. Furthermore, the $\Delta sit1$ mutant (lacking the siderophore transporter Sit1) could grow on ferritin plates. These results suggest that the mechanisms of iron utilisation from ferritin are independent of both siderophore uptake and the haemoglobin iron acquisition system.

The *RIM101* pathway of *C. albicans* is required for pH sensing and essential for an appropriate transcriptional response to changes in the environmental pH. Therefore, the relationship between pH, the reductive pathway and iron acquisition from ferritin was investigated further by analysing mutants lacking components of the *RIM101* pathway ($\Delta rim101$ and $\Delta dfg16$). These mutants had decreased ability to acquire iron from ferritin at physiological pH, but displayed no growth defect at acidic pH. Consistent with these findings, it has previously been demonstrated that both Rim101 and Dfg16 are required for growth in iron-limited media at alkaline but not acidic pH (Bensen *et al.*, 2004; Thewes *et al.*, 2007). Furthermore, it has recently been shown that the transcription factor Rim101 directly regulates the expression of two ferric reductases in *C. albicans* (*FRE2* and *FRP1*) (Baek and Davis, 2008). Therefore, it can be concluded that sensing the environmental pH and up-regulation of genes involved in the reductive pathway are necessary for iron acquisition from ferritin. The fact that ferritin iron utilisation is controlled by this pH-responsive transcriptional regulator, again underscores the importance of pH in iron acquisition from ferritin.

Because *S. cerevisiae* was not able to grow on ferritin plates at physiological pH, but still able to acidify the media when free iron was present (Figure 4.10), it was predicted that a close association between fungal cells and ferritin would be required for the release of iron from ferritin and subsequent uptake into the fungal cell. In agreement with this hypothesis, it was shown that *C. albicans* was able to bind ferritin at the cell surface. In addition, this binding event was hyphal-specific, since two mutants ($\Delta ras1$ and $\Delta cph1/efg1$) unable to form hyphae under the hyphal-inducing conditions used

(RPMI medium, 37°C and 5% CO₂) were also unable to bind ferritin. Furthermore, ferritin bound at the external cell wall layer and this binding was not regulated by the iron status of the fungal cell or the presence of iron inside the ferritin nanocage. Finally, the ferritin binding on the hyphal cell wall resulted in subsequent iron uptake from this molecule, as preincubation of *C. albicans* with ferritin (but not with apoferritin) permitted replication on agar plates with iron chelator (see 4.3.5). Similarly, a plethora of pathogenic microbes can utilise iron from host iron proteins, such as haemoglobin and transferrin, by binding of the iron-containing protein via specific surface receptors (Gerlach *et al.*, 1992; Pettersson *et al.*, 1994; Stojiljkovic *et al.*, 1995; Ekins *et al.*, 2004; Weissman and Kornitzer, 2004; Knight *et al.*, 2005; Torres *et al.*, 2006).

Theoretically, it is possible that a secondary unknown molecule is secreted by *C. albicans*, which binds ferritin and subsequently delivers the iron protein to a surface receptor – analogous to the situation in some bacteria which can secrete haemophores that bind extracellular haemoglobin and mediate its delivery to surface receptors (Genco and Dixon, 2001). However, it appears that such a scavenging mechanism is not involved in ferritin-binding by *C. albicans*, since fungal cells, killed with thimerosal – and then washed, removing any secreted factors – were still able to bind ferritin. Interestingly, fungal cells killed via exposure to UV-light lost their ability to bind ferritin. This result suggests that ferritin-binding at the cell surface is mediated by a protein receptor as it is known that certain proteins are inactivated by exposure to UV light (Voss *et al.*, 2007)

5.4. The ferritin receptor Als3

Several lines of evidence suggest that the GPI-anchored protein Als3 is a specific receptor which permits binding of ferritin and iron acquisition. (1) Ferritin-binding is blocked by exposure to UV-light, suggesting that the receptor is a protein. (2) Only hyphae, but not yeast cells bind ferritin and Als3 is known to be a hyphal-specific protein. (3) Mutants lacking factors known to regulate *ALS3* expression (Tec1, Bcr1) (Schweizer *et al.*, 2000; Nobile and Mitchell, 2005) have reduced abilities to bind ferritin. (4) A mutant lacking *ALS3* is completely unable to bind ferritin and an *Δals3+ALS3* revertant strain has a restored capacity to bind ferritin. Finally, (5) A *S. cerevisiae* strain expressing Als3 can bind ferritin.

The discovery that the $\Delta als3$ mutant completely lost its ability to bind ferritin was surprising considering that *ALS3* belongs to a gene family with several similar members. Although not every member of the Als family was investigated for ferritin binding, the fact that the *C. albicans* mutant lacking Als3 and *S. cerevisiae* strains expressing Als1 or Als5 (two other members of the *C. albicans* Als family) were unable to bind ferritin (while a *S. cerevisiae* strain expressing Als3 showed ferritin binding), suggests a unique property of Als3.

As discussed above, close contact between ferritin and the fungal cell was proposed to be necessary to exploit iron from ferritin. In fact, the Als3-mediated binding of ferritin to the fungal cell wall was essential for efficient exploitation of ferritin iron, as the $\Delta als3$ mutant – which displayed no defect in high-affinity iron uptake – grew poorly on agar plates with ferritin as the sole source of iron (Figure 4.27). Reconstitution of *ALS3* in the $\Delta als3$ mutant at least partially restored the ability to grow on such medium, although not to wild type levels. The inability to fully reconstitute the wild type phenotype may be due to a gene dosage effect or minor functional allelic variation, as has been described for other members of the Als family (Zhao *et al.*, 2007b).

5.5. The ability to exploit iron from ferritin during interaction with host cells

Binding of ferritin to hyphal surfaces not only occurred with exogenously added purified ferritin, but also during interactions of *C. albicans* with epithelial cells. *C. albicans* incubated with an oral epithelial monolayer showed accumulation of ferritin on the surface of the hyphal filaments. Furthermore, ferritin accumulation was predominantly observed on those hyphae which had invaded into epithelial cells; this observation is in agreement with the fact that ferritin is predominantly an intracellular iron storage protein. Finally, the hyphae of the $\Delta als3$ mutant did not display ferritin accumulation, while the $\Delta als3+ALS3$ revertant strain (like the wild type) had dense layers of ferritin on hyphal surfaces.

Although these data do not provide direct evidence that ferritin is used as an iron source during interaction with epithelial cells, they show that ferritin is in close contact to invading *C. albicans* hyphae and thus is available for exploitation by the proposed mechanism discussed above (5.3). This model is supported by the fact that mutants

which are unable to utilise ferritin *in vitro* ($\Delta als3$ and $\Delta afr1$) completely lost their ability to damage epithelial cell monolayers. Furthermore, the $\Delta als3$ mutant was significantly reduced in its ability to damage epithelial cells in the RHE model (Zhao *et al.*, 2004). However, it should be noted that Als3 has already been described as an adhesin (Zhao *et al.*, 2004) and invasin (Phan *et al.*, 2007). Therefore, the reduced ability of the $\Delta als3$ mutant to damage oral epithelial cells may be an accumulative effect, due to reduced adhesion, reduced invasion and/or reduced ability to use ferritin as an iron source.

5.6. Iron availability and virulence factors

Several studies have shown that pathogenic microbes link the availability of iron with virulence attributes (see 2.2.1). In this study, it was shown that a similar link between the regulation of an iron acquisition system and virulence attributes exists in *C. albicans*. In fact, the regulation of the ferritin receptor, Als3 is independent of external iron sources and seems to be strictly linked to hyphal formation. Therefore, iron acquisition from the intracellular iron storage protein ferritin is hyphal regulated. Hyphal formation itself however is associated with adhesion, proteolytic activity, cellular invasion and damage (Sundstrom, 2002; Naglik *et al.*, 2004; Phan *et al.*, 2007; Thewes *et al.*, 2007; Zakikhany *et al.*, 2007) and hyphae are those fungal morphologies which predominantly reach the intracellular compartments of epithelial cells where ferritin is located. Therefore, during interactions with oral epithelial cells, *C. albicans* co-regulates morphology, invasion, tissue damage and an iron acquisition system. This view may explain why iron acquisition from ferritin is a hyphal-specific property and does not occur with the normally non-invasive yeast cells.

5.7. Environmental challenges and hyphal-associated proteins

As discussed above, iron acquisition from the intracellular iron storage protein ferritin is co-regulated with hyphal development under the experimental conditions used in this study. However, *C. albicans* is a highly flexible pathogen which can adapt to a multitude of challenges encountered in the host. Sosinska and co-workers recently

observed that hypoxic conditions and iron restriction in a vagina-simulative medium affected cell morphology and the cell wall proteome of *C. albicans*. Interestingly, one of the proteins found in the cell wall of yeast cells under these iron limited conditions was Als3 (Sosinska *et al.*, 2008). This result demonstrates hyphal-independent expression of Als3, possibly via iron starvation, providing further evidence for the role of Als3 in iron acquisition.

Moreover, a mutant lacking Hgc1, a hyphal-specific G1 cyclin-related protein (Zheng *et al.*, 2004), does not produce true hyphae in hyphal inducing-conditions. Under these conditions however, $\Delta hgc1$ mutant cells still express *ALS3* at the same level as the wild type (see Table 4.2) and consequently are still able to bind ferritin. Thus, ferritin binding does not require hyphal morphology *per se*. The expression of *ALS3* in $\Delta hgc1$ cells, demonstrated in this study, is consistent with the findings of Zheng and co-workers, who initially showed that the $\Delta hgc1$ mutant retains normal expression levels of three hyphal-associated genes (*HWPI*, *ECE1* and *HYR1*) (Zheng *et al.*, 2004). Whether-or-not the products of these genes are functional, or even correctly localised in the $\Delta hgc1$ mutant, has not yet been reported. Thus, the current study shows for the first time that a hyphal-associated gene (*ALS3*) is not only expressed in the $\Delta hgc1$ mutant, but also that the gene-product is correctly localised to the cell surface, where it retains its function as a ferritin receptor. Moreover, White and co-workers have recently shown that *C. albicans* expresses a number of hyphal-specific genes (such as *ECE1*) in a murine gut model of commensalism, whilst growing in the yeast morphology.

According to the data above, it can be concluded that under specific environmental conditions – especially within a host – *C. albicans* is able to dramatically remodel its transcriptome. For example, yeast cells can express genes that were previously thought (based on expression analysis during laboratory growth conditions) to be hyphal-specific genes. It is therefore important to consider the transcriptome of *C. albicans* as a function of the specific niche which the fungus occupies, rather than the gross morphology of the cells observed.

5.8. Multiple functions for Als3

The Als protein family of *C. albicans* encodes large cell surface GPI-glycoproteins that were originally implicated in the process of adhesion to host surfaces by the group of Hoyer (Hoyer, 2001). Expression of *ALS3* was shown to be hyphal-specific (Hoyer *et al.*, 1998) and was observed *in vivo* during oral and systemic infections (Thewes *et al.*, 2007; Zakikhany *et al.*, 2007). In addition to its adhesion properties, Als3 was recently shown to be an invasin that binds to cadherins and induces endocytosis by host cells (Phan *et al.*, 2007). In this study, it was observed that Als3 possesses yet another function, mediating iron acquisition via highly specific binding to host ferritin, indicating that this single protein has multiple pathogenicity-associated functions.

Surprisingly, a mutant lacking Als3 displayed wild type virulence in a mouse model of systemic candidosis (Scott Filler, personal communication). Because Als3 is a protein with many pathogenicity-associated properties, the fungus must somehow bypass the lack of Als3. For example, it is likely that the lack of Als3 – in the context of iron acquisition – is compensated by other iron acquisition systems and that non-ferritin iron sources are used by the $\Delta als3$ mutant during systemic infection. A similar explanation has recently been suggested by Weissman and Kornitzer when discussing the fact that a $\Delta rbt5$ mutant lacking a haemoglobin receptor was as virulent as the wild type in a mouse model of systemic infection (Weissman and Kornitzer, 2004). Secondly, the lack of the adhesion properties of Als3 in the $\Delta als3$ mutant may be compensated *in vivo* by upregulation of other members of the Als family, Hwp1 or other as-yet unidentified adhesins. Thirdly, the lack of the invasin properties of Als3 in the $\Delta als3$ mutant may be compensated *in vivo* by Als1, since Als1 and Als3 can both mimic host cell E- and N-cadherins (Phan *et al.*, 2007). Similarly, Nobile and co-workers showed that Als3 was required for biofilm formation *in vitro*, but not *in vivo* (Nobile *et al.*, 2006). The authors argued that additional adhesins can partially compensate for the absence of Als3 *in vivo* but not *in vitro*, due to higher expression levels of the compensatory adhesins *in vivo* compared to the *in vitro* situation.

5.9. The iron permease Ftr1 is required for iron acquisition from two different host iron proteins

As shown in this thesis, both the high-affinity iron reductive pathway and binding are crucial for ferritin iron acquisition by *C. albicans*. Similarly, binding to transferrin, together with the reductive pathway, was shown to be necessary for iron utilisation from transferrin by this fungus (Knight *et al.*, 2005). In these studies, a mutant lacking the iron permease Ftr1, which is an essential component of the reductive pathway, was unable to grow with either ferritin (see 4.2.5) or transferrin (Knight *et al.*, 2005) as the sole source of iron. The same mutant lost its ability to damage oral epithelial cells (see 4.5.4) and was completely avirulent in a mouse model of systemic infection (Ramanan and Wang, 2000). These data suggest that Ftr1 is involved in iron acquisition from at least two different host proteins, making this permease essential for *C. albicans* virulence and an attractive target for drug development.

5.10. The “glove effect” phenomenon and immune cell evasion

During the immunofluorescence analysis of interactions between *C. albicans* and oral epithelial cells, it was observed that the intracellular hyphae were able to outwardly stretch epithelial cell membranes; this process resembled a finger within a glove and so termed the “glove effect”. The molecular mechanisms governing this phenomenon remain to be investigated. However, it was observed that the iron status of the epithelial cells plays a role in this process since no “glove effect” phenomenon was observed in ferritin-depleted oral epithelial cells co-incubated with *C. albicans*. One possible explanation is that an excess of intracellular host iron increases lipid peroxidation, making the plasma membranes more flexible under hyphal pressures.

Invasive bacteria, such as *Listeria monocytogenes*, actively induce their own uptake by endocytosis and then disseminate from cell to cell by means of an actin-based motility process (Cossart and Sansonetti, 2004). *C. albicans* can also induce its own endocytosis by host cells (Filler and Sheppard, 2006). In addition to endocytosis, the fungus can actively penetrate into host cells (Zakikhany *et al.*, 2007). Hyphal formation is necessary for both endocytosis and active penetration. In contrast to invasive bacteria,

C. albicans, through hyphal elongation, was able to escape from one epithelial cell, reach the extracellular space and subsequently invade the neighbouring host cell.

It is known that neutrophils, as essential cells of the host innate immune response to fungi, preferentially attack *C. albicans* hyphae, rather than yeast cells (Wozniok *et al.*, 2008). Thus, it can be speculated that *C. albicans* hyphae hide from immune cells (such as neutrophils) within the epithelial cells. Therefore, the observed “glove effect” may be a mechanism to traverse the intercellular distance between two host cells without the fungus exposing itself to the immune system.

5.11. Conclusions and Outlook

Data obtained in this thesis provide sufficient information to construct an attractive and comprehensive model of iron utilisation from ferritin by *C. albicans*. Taken as a whole, the data presented here suggest that ferritin is used as an iron source by *C. albicans*. The fungus is able to bind ferritin via a direct interaction with Als3 on the surface of hyphae. Next, the ferritin iron is made available through acidification of the surrounding environment; secretion of reductants may also play a role in the release of iron from ferritin. Finally, the released iron is transported into the fungal cell via the reductive pathway (Figure 5.1).

Schematic of hyphal cell surface

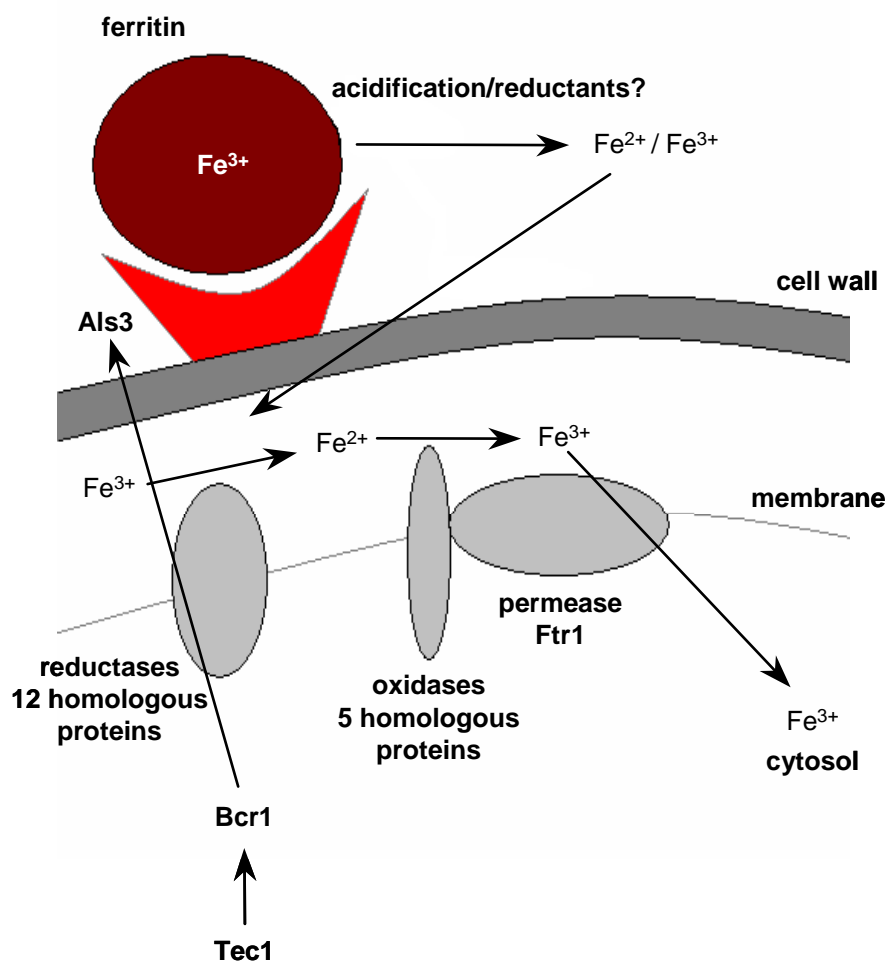


Figure 5.1. Proposed model for iron utilisation from ferritin by *C. albicans*

Ferritin is a novel iron source used by *C. albicans*. In its hyphal form, *C. albicans* binds ferritin using Als3. Through acidification/reductants, the iron molecules are released from the ferritin shell and transported into the cell via the reductive pathway.

Although this study met its aims in identifying and characterising a novel iron source for *C. albicans* during oral infection, the findings presented in this thesis open up avenues for future research.

- It was shown that *C. albicans* possesses intracellular iron storage. However, nothing is known about the molecular mechanisms involved in this process. In *A. fumigatus*, the siderophores involved in intracellular iron storage are required for germ tube formation, asexual sporulation, resistance to oxidative stress, catalase A activity and virulence (Schrettl *et al.*, 2007). A better understanding of iron storage in *C. albicans* may shed

light on intracellular iron regulation and its involvement in the virulence of this important human pathogen.

- Acidification of the surrounding environment is important for iron acquisition from ferritin by *C. albicans*. As acidification may also be beneficial for a number of other properties (such as activity of aspartic proteases [Naglik *et al*, 2003]), it would be of importance to show whether such acidification occurs *in vivo*.
- The possible involvement of secreted reductants in the iron acquisition process remains to be investigated. For example, *C. neoformans* secretes 3-hydroxyanthranilate (a reductant), which is involved in transferrin iron exploitation by this fungus (Jacobson *et al.*, 2003). Questions which could be answered in future studies are: (i) Can *C. albicans* produce and secrete reductants? (ii) What kind of reductants can be produced by this fungus? (iii) Are these reductants involved in ferritin iron acquisition? (iv) Do these molecules play a role in the pathogenicity of *C. albicans*?
- Phan and co-workers have demonstrated that an N-terminal domain of Als3 binds to N- and E-cadherins to induces *C. albicans* endocytosis by host cells (Phan *et al.*, 2007). However, the ferritin-binding domain of Als3 remains unknown. By selectively deleting specific regions of the *ALS3* gene, together with immunofluorescent tagging and localisation studies, it should be possible to functionally characterise the domains of Als3 responsible for its diverse functions (ferritin binding, adhesion, invasion).
- The fact that the iron permease Ftr1 is required for iron acquisition from at least two host iron proteins and for virulence makes this permease an ideal drug target. Future research towards the development of a molecule capable of specifically blocking the permease channel of Ftr1 could provide a novel therapeutic for the treatment of candidosis.
- Although the “glove effect” phenomenon observed in this study was shown to be iron-dependent, the molecular mechanisms underlying this process remain unknown. A better understanding of this process may provide insights into the largely unknown pathogenesis of superficial *Candida* infections and thus may deliver new therapeutic strategies.

6. References

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7. List of Abbreviations

BPS	Bathophenanthrolineisulfonic acid disodium salt
BSA	Bovine Serum Albumin
CIp10	<i>Candida</i> Integration plasmid 10
DIC	Differential Interference Contrast
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
FBS	Fetal Bovine Serum
g	gram
h	hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
kDa	kilo Dalton
l	litre
LDH	Lactate Dehydrogenase
LIM	Limited Iron Medium
M	Molar
mg	milligram
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
mRNA	messenger-RNA
NCBI	National Center for Biotechnology Information
ng	nanogram
nm	nanometre
orf/ORF	Open Reading Frame
PBS	Phosphate-Buffered Saline without calcium or magnesium
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
rpm	rotation per minute
s	second
SD	Synthetic Dextrose medium
SDS	Sodium Dodecyl Sulfate
spp.	species
Tris	Tris(hydroxymethyl)aminomethane
UV	Ultraviolet
YNB	Yeast Nitrogen Base
YPD	Yeast-extract Peptone Dextrose medium
µg	microgram
µl	microlitre

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